Role and Regulation of Phosphaturic Hormones in Autosomal Dominant Polycystic Kidney Disease

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1 Zusammenfassung

Die autosomal dominante polyzystische Nierenerkrankung (ADPKD) ist eine genetische Erkrankung, welche charakterisiert ist durch die Zerstörung der Nierenarchitektur und den Verlust der renalen Funktion auf Grund progressiver bilateraler Ausbildung zahlreicher Zysten. Es konnte gezeigt werden, dass die Ausbildung solcher Zysten bereits sehr früh im Uterus der Mutter beginnt, so dass zwischen dem 18. und 40. Lebensjahr 50% des normalen Nierenparenchyms durch Zysten ersetzt sind. Bei den meisten ADPKD Patienten wird die glomeruläre Filtrationsrate (GFR) der erkrankten Nieren durch kompensatorische Hyperfiltration bis ca. zur 4. Lebensdekade aufrechterhalten. Die Hyperfiltration basiert auf der Gegenreaktion verbleibender funktionstüchtiger Nephrone, den fortschreitenden Funktionsverlust des Nierengewebes auszugleichen. Die polyzystische Nierenerkrankung kann früh in ihrem Verlauf diagnostiziert werden, und stellt ein ideales Modell dar, um den Mineralstoffhaushalt in frühen Stadien einer chronischen Nierenerkrankung („chronic kidney disease“, CKD) zu studieren. In der vorliegenden Arbeit wurde die ADPKD als Modellkrankheit für CKD Stadium 1 (GFR > 90 ml/min/1.72 m^2) und 2 (GFR 60-90 ml/min/1.72 m^2) gewählt, um den Kalzium-Phosphat–Haushalt und die Wirkung von 4 hormonalen Faktoren (Fibroblast Growth Factor 23 [FGF23], Vitamin D, Parathormon [PTH] und Klotho) zu ergründen.

FGF23 ist ein phosphaturisches Hormone welches von den Osteozyten ausgeschüttet wird. Erhöhte Mengen dieses 30 kDa schweren Peptids wurden sowohl im Serum von Patienten mit Tumor-induzierter Osteomalazie (TIO) gefunden, als auch bei Patienten mit anderen genetischen Erkrankungen wie der autosomal dominanten hypophosphatämischen Rachitis (ADHR) basierend auf einer Mutation des Gens welches für FGF23 kodiert, der autosomal rezessiven hypophosphatämischen Rachitis (ARHR) basierend auf einer Inaktivierungsmutation des Dentin Matrix Acidic Phosphoprotein (DMP1), und bei X-linked

Hypophosphatemia (XLH) basierend auf einer Inaktivierungsmutation von PHEX, welches dazu beiträgt, dass FGF23 vor Degradierung abgeschirmt wird. Bei all diesen Erkrankungen ist die Serumkonzentration von FGF23 erhöht und führt zu massiver renaler Phosphatausscheidung. Bei Patienten mit chronischer Nierenerkrankung im Stadium 1 und 2 trägt die beginnende Erhöhung von FGF23 im Serum dazu bei, den Phosphatgehalt im Blut im Normbereich zu halten. Unsere Resultate bei Patienten mit ADPKD mit CKD 1 und 2 zeigten interessanterweise bezüglich FGF23 ein anderes Bild als bei Patienten mit anderen Nierenerkrankungen. ADPKD Patienten zeigten im Vergleich mit anderen CKD Patienten massiv erhöhte FGF23 Werte im Serum. Ähnlich wie bei Patienten mit TIO, ADHR, ARHR oder XLH, waren die Konzentrationen von FGF23 auch bei normaler GFR erhöht. Ein grosser Unterschied zu den genannten Erkrankung war jedoch, dass trotz ähnlichen FGF23 Serumspiegeln keine Hypophosphatämie bei den ADPKD Patienten vorlag, was auf eine Resistenz auf FGF23 schliessen lässt.

Klotho ist der hauptsächlichste Rezeptor von FGF23. Dieses 130 kDa schwere Protein hat eine einzelne transmembrane Domäne und ist hauptsächlich in der Niere, der Nebenschilddrüsen und der Choroidea im Auge exprimiert. Sobald Klotho vom Endosom zur Zellmembran transloziert, wird die extrazelluläre Domäne von Klotho (solubles α-Klotho) abgetrennt und in den Blutkreislauf freigesetzt. FGF23 benötigt die c-terminale Bindung zu Klotho um den kanonischen Rezeptor FGFR1c zu aktivieren. Diese Aktivierung wiederum führt in proximalen Tubuluszellen der Niere zur verminderten Membranexpression der NaPiIIa und NaPiIIc Phosphatkotransporter, wie auch zur Verringerung der 1α-hydroxylase Expression und Aktivität. Da bei Patienten mit ADPKD die FGF23 Serumkonzentrationen massiv erhöht sind, analysierten wir die Serumspiegel von solublem α-Klotho bei ADPKD Patienten und verglichen sie mit Patienten derselben CKD Stadienmit anderen Grundkrankheiten. Diese Analyse zeigte, dass bei ADPKD Patienten die Konzentration von zirkulierendem α-Klotho stark vermindert ist. Basierend auf diesem Resultat vermuten wir,

dass die periphere Resistenz auf FGF23 mit der tiefen Expression von zirkulierendem α-Klotho begründet werden kann.

Zusammenfassend lässt sich festhalten, dass die spezifische Erhöhung von FGF23 in frühen CKD Stadien und die assoziierten niedrigen Klotho Werte eine spezifische Manifestation der polyzystischen Nierenerkrankung darzustellen. Diese Veränderungen könnten auch mit der Zystenbildung verknüpft sein. Alternativ könnte die Entwicklung der Zysten und die darauffolgende Zerstörung des Nierenparenchymszur niedrigen Expression von Klotho beitragen. Viele Zysten bilden sich im distalen Tubulussegment, welches auch die Hauptlokalisation der Transmembranexpression von Klotho ist. Die pathophysiologische Bedeutung der veränderten FGF23 und Klotho Expression und deren Auswirkungen auf den Mineralhaushalt bei Patienten mit ADPKD muss in weiteren Studien geklärt werden.

2 Summary

Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disorder characterized by the destruction of the kidney architecture and loss of renal function due to progressive bilateral growth of numerous cysts.[1, 2] It has been demonstrated that the development of cysts is a process that starts already early in uterine, thus by the age between 18 and 40 years the cysts replace approximately 50% of the normal parenchyma.[3]

In most ADPKD patients, glomerular filtration rate (GFR) of the affected kidney remains preserved up to the 4th decade due to a compensatory glomerular hyperfiltration in the remaining nephrons counteracting the ongoing loss of functional renal tissue.[4, 5] ADPKD can easily be detected early in the course of the disease and represents an ideal condition to study mineral metabolism at an early stage of chronic kidney disease (CKD) since GFR remains stable for a long time and the course of the disease is generally not confounded by therapies. Using ADPKD as an optimal model to study early onsets of CKD stage 1 (GFR > 90 ml/min/1.72 m^2) and stage 2 (GFR 60-90 ml/min/1.72 m^2), we mainly focused on the phosphate / calcium homeostasis and involvement of the 4 major hormonal players influencing the mineral metabolism of CKD (Fibroblast Growth Factor 23 [FGF23], Vitamin D, Parathormon [PTH] and Klotho).

Firstly, FGF23 is a known as a phosphaturic hormone that is secreted by osteocytes.[6, 7] Serum levels of this 30 kD peptide were found to be elevated in tumor induced osteomalacia (TIO)[8, 9] as well as in several genetic diseases such as autosomal dominant hypophosphatemic rickets (ADHR) secondary to a mutation of the gene which encodes for FGF23,[10, 11] autosomal recessive hypophosphatemic rickets (ARHR) secondary to an inactivating mutation of Dentin Matrix Acidic Phosphoprotein (DMP1),[12, 13] and X-linked hypophosphatemia (XLH) secondary to inactivating mutations of PHEX,[14] which makes FGF23 less accessible to

degradation. In disease states where FGF elevation has been described, its accumulation led to severe renal phosphate wasting. In contrast, elevated FGF23 levels in CKD patients may play an essential role in maintaining normal serum phosphate levels at CKD stages 1 and 2.[15-17] Our first finding, using ADPKD as a model for early CKD, was different to other diseases inducing kidney insufficiency. ADPKD presented with significantly higher expression levels of FGF23, when compared to CKD patients not affected with polycystic kidney disease .[18] Similar to TIO, ADHR, ARHR and XLH, this finding in ADPKD patients occurred even in the presence of a normal GFR. However, the marked hypophosphatemia, typical for tubular disorders is not present in ADPKD, suggesting a resistance to FGF23 in this disease.

Klotho has been determined to be the main co – factor for FGF23. The gene Klotho encodes for a 130 kD protein with a single transmembrane domain that is mainly expressed in the kidney, the parathyroid glands and the choroid plexus.[19] Once Klotho protein is translocated from the endosome to the cell membrane, the extracellular domain of Klotho (soluble α-Klotho) may be cleaved and released into the blood stream.[20] FGF23 needs to bind Klotho protein by its c-terminal part to activate the canonical receptor FGFR1c which leads to reduced membrane expression of the NaPiIIa and NaPiIIc co-transporters as well as down regulation of 1-alpha hydroxylase.[21] Subsequently to the finding that FGF23 is markedly elevated in ADPKD, we analyzed other CKD patients not affected with polycystic kidney disease yet in similar CKD stages, and established that our ADPKD patients present significantly lower levels of Klotho when compared to non polycystic CKD patients. Therefore we concluded that this results pointed toward the hypothesis that peripheral

resistance to FGF23 could be triggered by lower local expression or circulating levels of Klotho.

In summary we established that the specific elevation of FGF23 in early stages and the associated decrease of Klotho is a manifestation specific for polycystic Kidney disease and might be implicated in the development of cysts. Whereas alternatively cyst development and the subsequent destruction of the epithelial parenchyma may contribute to the low levels of Klotho expression, especially since the origin of cyst development is considered to be the distal convoluted tubule, the location of the Klotho transmembrane molecule expression.

Yet, the pathophysiological importance of level alterations of FGF23 and Klotho and the associated implication on the mineral homeostasis of ADPKD patients is subject to further research efforts. [22]

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4 Autosomal Dominant Polycystic Kidney Disease

4.1.1 Genetic Mutations and Phenotype

Autosomal dominant polycystic kidney disease (ADPKD) is a common, monogenic multi-systemic disorder characterized by the development of renal cysts leading to a subsequent complete destruction of the renal parenchyma in the fifth or sixth decade of a human life.[23, 24] Its autosomal dominant characteristics manifest due to the fact that in case one parent carries the disease, there is a 50% chance that the disease will be passed on to the child, therefore will be inherited dominantly throughout the generations.

It has a prevalence of 1: 400 to 1: 1000 in white populations and is accounted for 10 – 15% of the end stage renal disease cases leading to mandatory maintenance on dialysis.[25] In 85% of the ADPKD cases the underlying genetic mutation is located on the chromosome 16 linked on a α-globulin complex, defined as the polycystic kidney disease 1 gene (PKD 1) that encodes for the protein polycystin 1 (PC1)[26]. The gene product is a membrane bound protein with a large extracellular structure containing a C type lectin domain, leucin rich and immunoglobulin like repeats and type III fibronectin domains.[27] This structure suggest that PC1 can mediate cell – cell and cell-matrix interaction.[27] The majority of the remaining cases are associated with a defect on chromosome 4 defined as polycystic kidney disease 2 gene (PKD2), encoding for polycystin 2 (PC2)[28], which is as well a membrane bound protein that shows an integral component of an ion channel complex.[29] The cases that are determined to be caused solely due to a mutation in PKD2 are found to be less severe than the phenotypes

based on the PKD1 mutations.[28, 30] Earlier on it has been postulated that PC1 is a regulator of PC2, and that both proteins are involved in a signaling pathway that is essential for normal cytogenesis and renal tubulogenesis. This phenotypic variability is based on the PKD genic and allelic variability and ability to act as modifier genes, where the proteins encoded by these genes are involved in mechanosensory ion channel complexes and integrity of the extracellular matrix.[24, 31] Cyst development therefore is caused by somatic mutations in the PKD genes leading to loss heterozygosity (LOH).[32] In fact, one hypothesis persisting today, states that the mutation found in the germline is not in itself sufficient to produce a cyst, and a second sporadic event, genetic or otherwise, is also required (two hit model).[33] It is actually accepted that the second hit necessary for cysts formation inactivates the wildtype copy of the allele, which is inherited from the healthy parent[34]. Although the exact mechanism of cyst development still builds on postulations that remain to be confirmed, it has been agreed upon that most of the cysts arise first in the nephron from the loops of Henle, distal tubules and collecting ducts.[35, 36]

4.1.2 Cyst formation, Phenotypes and Organ Manifestations

The pathogenesis of ADPKD has been divided into three phases. The initiation phase where normal quiescent epithelial cells become hyperproliferative, the cyst growth phase and the cyst expansion phase caused by a proliferation of cyst epithelial cells and increased fluid secretion, as well as extracellular metric synthesis.[37] Additional signaling pathways that under normal circumstances regulate cyto- and tubulogenesis during the phase of cell proliferation

are shown to be highly dysfunctional in ADPKD causing the typical formation of cysts.[26] At a certain point in the cyst growth phase the cyst detaches from the original nephron and continues to grow isolated and autonomous.[38]

ADPKD patients form renal cysts as described focally from the nephron, current research focus on the hypothesis that the beginning of the cyst formation is a process that starts in the loop of Henle and the distal convoluted tubules.[36] An additional manifestation of the ADPKD disease state is the cyst development in the intrahepatic biliary tree, where epithelial cells from these independent cysts are monoclonally derived.[39] Development of hepatic cysts is a common manifestation along the disease progression of ADPKD and happens generally after the development of renal cysts, hence later in the disease state. Liver cysts are responsible for most hepatic complications, where as in the later stage of ADPKD liver changes may occasionally be encountered, including congenital hepatic fibrosis and segmental dilation of the biliary tract.[39] The prevalence and number of hepatic cysts in patients with ADPKD increase with progression of age, female gender, severity of renal cystic disease, and severity of decline of renal function. [40] By age 60, nearly 80% of patients have hepatic cysts. Additional manifestations of ADPKD are cerebral aneurysms, pancreatic cysts, cardiac valve disease, colonic diverticula, abdominal wall, hypertension and inguinal hernia. Malformations of selected vasculature, including intracranial aneurysms and aortic root dilatation may be due to altered expression and function of the PKD gene in arterial smooth muscle cells and myofibroblasts[41].

Historically one of the main indications if an individual is at risk to develop cystic kidney disease was based on family history, since the mutation is inherited dominantly and has a 50% chance to be carried on if one parental carrier exists. New mutations account for only 3.6% of cases, where family history is not always positive[23]. Current diagnostic methods involve magnetic resonance imaging (MRI) and Ultrasound evaluation including the proper analysis of family history[42] and screening for mutations in the PKD gene via genetic testing, yet the latter option is considered limited due to methodological restrictions. Therefore it is of essence to individualize the patient care and screening in order to get a complete picture of the status quo and a possible forecast on ADPKD development.

4.1.3 ADPKD – preserved CKD 1 stage due to hyperfiltration

ADPKD is a disease that has its onsets already in uterus and progresses slowly with the development form early childhood to adulthood and further.[43] Due to its slow progression and the diagnostic imaging tools and the proper screening regarding family history, a final diagnosis of APDKD can be provided already in early adulthood.[42] The affected patients present with preserved kidney function due to compensatory hyperfiltration, that can be defined as chronic kidney disease (CKD) stages 1 and 2.[41] In the case of ADPKD, hyperfiltration is a state where the remaining functionally not affected nephrons go into overcompensation to maintain the required glomerular filtration rate of the kidney.[41] Diagnostic markers for ADPKD can be decreased effective renal plasma flow (ERPF), increased filtration fraction (FF) and urinary albumin excretion (UAE) and finally enlarged total renal volume (TRV). These parameters may be better or more so additional markers for

disease severity in combination with the overall glomerular filtration rate (GFR).[44] As stated earlier; despite this significant organ dysfunction, the preserved and maintained filtration for a prolonged period of time, makes ADPKD an appropriate model to study mineral ion homeostasis in early renal disease, defined as CKD stage 1 and 2 (eGFR > 60ml/min). [44]

5 Mineral Ion Metabolism in Chronic Kidney Disease

5.1 Calcium & Phosphate Homeostasis

5.1.1 Regulations and Transport of Phosphate

The disruption and dysregulation of phosphate homeostasis happens early in the course of chronic kidney disease. Accompanied by a decrease in calcitriol production and elevation of parathyroid hormone (PTH) levels, the derangements of mineral ion homeostasis and hormone regulation characterize the manifestation of secondary hyperparathyroidism (sHPT), while defining the disease state of chronic kidney failure.[45]

In a healthy person, serum phosphate concentration is maintained within a narrow range (0.8 – 1.5mmol/L), despite variable amounts of phosphate that is daily ingested. 30% of the total phosphate ingested by nutrients is excreted through the gastrointestinal tract and 70%, which accounts for approximately 700 mg/day, is excreted by the kidneys. In relation to a normal GFR, 15% of the filtered load of phosphate must be excreted; the tubular reabsorption of phosphate is equal to about 85% of the filtered load. The absolute value for tubular reabsorption of phosphate will vary depending upon the amount of phosphate requiring

excretion. The mechanisms controlling phosphate homeostasis are greatly depending on various effectors that influence and regulate phosphate homeostasis.[46] The molecular mechanism regarding phosphate excretion and reabsorption in the proximal tubule is mediated by Na-dependent, secondary-active transport mechanisms (Na/Pi II transporters). The influx of phosphate from the tubular lumen into the epithelial cell involves brush border membrane-associated Na/Pi co-transporters which are rate-limiting and targets for physiologic/pathophysiologic regulation. [47-49] Three types of Na/Pi co-transporters have been identified. Characterization of the three different types varies among the localizations along the proximal tubule. The type I Na/Pi co-transporter is localized in the brush border membrane of proximal tubular cells.[50, 51] It still remains to be clarified if this transporter is essential to facilitate phosphate uptake. Current opinion is that type I mediated Na/Pi cotransport does not have the characteristics and regulatory features of brush border membrane Na/Pi cotransport and suggested a channel function, mediating the flux of chloride and organic anionic compounds. Two closely related type II Na/Pi co-transporters are expressed in the apical membrane of absorptive/reabsorptive epithelia, the type IIa[52], type IIb[53], and type IIc[52]. Type IIa and type IIc are majorly expressed in kidneys, but neither of them could be detected in the intestine. Type IIb is expressed in small intestine and type II alveolar cells, but not in kidney.[54, 55]

This finding makes the Na/Pi co-transporter Type IIa and c the essential phosphate transporter mechanism in the renal cells along the proximal tubules.[55-57]

Yet there is another phosphate transport system worth mentioning, Pit1 and Pit2 were identified as the mammalian representatives of type III sodium-phosphate symporters (NaP_i-III), these NaP_i-III transporters do not share homology or similarity with the above described phosphate co – transporters. At least Pit2 contributes also to renal phosphate reabsorption, is localized in the proximal tubular brush border membrane, and regulated by phosphate and PTH (Ravera..Murer..Sorribas et al 2008 oder 2009, Picard, Capuano…Wagner 2010).

5.1.2 Regulation and Transport of Calcium

Similar to phosphate, it has been determined that calcium shows level imbalance as soon as renal failure occurs. A homeostatic system works to ensure that the concentration of extracellular calcium is tightly controlled. Under normal conditions (1.1 – 1.4 mmol/L), serum ionized calcium fluctuates by a maximum of ± 2%.[58] An increased concentration of free calcium not bound to protein in serum, defined as ionized calcium (Ca^{2+}) is used as a key signaling messenger in the initialization of a wide range of cellular events. Ca^{2+} signals control many cellular functions ranging from short-term responses such as muscle contraction and hormonal secretion, such as secretion of parathyroid hormone, to long-term regulation of cell growth and proliferation.[59] It has been shown that Ca^{2+} signaling can induce apoptosis or less specific necrosis under special conditions.[60]

Calcium enters the cells by any of the general classes of channels, including voltage operated channels, second messenger-operated channels, store-operated channels and receptor-operated

channels. In non-excitable cells, not participating in the triggering of an action potential, the major Ca^{2+} entry pathway are store-operated channels, in which the emptying of intracellular Ca^{2+} stores activates Ca^{2+} influx. The increase in Ca^{2+} levels has been associated with cellular dysfunctions in a variety of conditions such as diabetes mellitus, hypertension, hyperparathyroidism and chronic renal failure.[61, 62] Furthermore, dysregulation of Ca^{2+} homeostasis involving the endoplasmic reticulum and store-operated calcium channels has been manifested in neurodegenerative disorders such as Alzheimer's patients[63], in patients with immunodeficiency, acute pancreatitis[64] and polycystic kidney disease.[65] These processes are dependent on the regulatory function of calciotropic hormones and their effector tissues in the kidney, intestine, and bone. Essential for this regulation mechanism is the calcium-PTH axis. Extracellular calcium levels are the primary marker of PTH secretion and production and, hence, PTH is a key regulator of serum calcium levels.[66]

5.2 Hormonal Control of Phosphate and Calcium Homeostasis

5.2.1 PTH and Vitamin D

The principal function of PTH is to keep blood Ca^{2+} constant by releasing calcium from bone, reducing renal excretion of calcium, increasing urinary excretion of phosphorus, and stimulate the production of the active form of vitamin D (calcitriol) in the kidneys. Vitamin D and the vitamin D receptors (VDRs) are expressed within the nucleus of parathyroid cells and play important roles in calcium homeostasis. The kidneys are able to respond to PTH by

reabsorbing calcium, excreting phosphorus, and increasing production of calcitriol, making PTH one of the regulatory factors for renal physiology.[67, 68] In the progression of CKD, increased PTH levels are one of the manifestations of secondary hyperparathyroidism (sHPT), causing increased proliferation of parathyroid gland tissue to cover the demand for sequestering PTH. This increased demand is on one hand due to high Pi and low Ca^{2+} based on the calcium phosphate product (CaxP) insolubility, on the other hand due to skeletal resistance to the action of PTH; which is postulated to be caused by down regulation of PTH receptors in bone cells, increased levels of osteoprotegerin and possible decreased levels of bone morphogenetic proteins.[69] In human physiology PTH levels are required to mobilize bone calcium in order to maintain normal bone turnover and calcium homeostasis. This so called bone turnover or bone remodeling is done by bone remodeling units of osteoblasts and osteoclasts, coupled together via a paracrine cell signaling. The process of bone resorption by the osteoclasts releases stored calcium into the systemic circulation therefore regulates calcium balance, making PTH a key factor in bone physiology.

Furthermore, and due to its regulatory involvement regarding the development of CKD, PTH has become along with phosphate and calcium the most important screening factor in daily clinical practice, and its maintenance and control has become one of the most challenging tasks for a physician.[70]

Along with PTH, vitamin D plays a pivotal role in the regulation of calcium–phosphate homeostasis. Vitamin D deficiency is a significant risk factor for the development of specific chronic diseases.[71] In CKD, vitamin D deficiency, specifically low calcitriol levels, is highly

prevalent and associated with sHPT, leading to serious mineral and bone disorders.[72] Many currently applied guidelines have determined for Vitamin D to be supplemented already in patients determined to be CKD stage 3 (National Kidney Foundation (K/DOQI) guidelines). The treatment of CKD patients with Vitamin D sterols or analogues has been well established in daily clinical practice. Both the supplementation of Vitamin D and the control of PTH have been well established treatment targets, yet the long term consequences and the involvement of and dependence on different hormonal regulators and effectors are still to be determined.

6 Phosphaturic Hormones

6.1 Fibroblast Growth Factor Family

The fibroblast growth factor (FGF) family consists of 22 individual members ranging from FGF1 – FGF23, all with a variety of functions. Human FGFs contain 150–300 amino acids and have a conserved core of 120 amino acids with 30–60% identity to each other.[73-75]

The FGF23 gene encodes for an approximately 32-kD (251 amino acids) protein with an N-terminal region that contains the FGF homology domain and a novel 71–amino acid C-terminus that was originally discovered by homology-based PCR screening of a mouse embryonic cDNA library. The FGF23 gene is located on Chr 12p13 and is, due to its genetic and amino acidic identity of 24%, genetically grouped with the FGF19 and 21 genes.[76 77]

Based on their function as growth factors, the FGFs family members are defined to act as hormones either classified as intracrine, paracrine and endocrine FGFs according to their

mechanisms of action. Intracrine FGFs, FGF11-FGF14, are not secreted extracellulary. They act as intracellular molecules in an FGF receptor-independent manner.[78, 79] They interact with intracellular domains of voltage gated sodium channels and with a neuronal MAPK scaffold protein. The only known role for intracrine FGFs is the regulation of the electrical excitability of neurons and possibly other cell types.[80, 81]

The remaining FGFs members are determined to act as paracrine hormones and are mostly secreted proteins with cleavable N-termini that act as secreted signal peptides. Except for FGF1 and FGF2, which have no N-terminal hydrophobic sequences, which is atypical for secreted proteins. FGF1 and FGF2 are postulated to be released from damaged cells or by an exocytic mechanism that is independent of the endoplasmic reticulum-Golgi pathway.[82]

The paracrine FGFs, which mediate biological responses as extracellular proteins that are known to have a heparin binding site to and activating cell surface tyrosine kinase FGF Receptor (FGFR) with heparin/heparan sulphate as a cofactor.[83] The FGFR genes have been categorized as 4 different types FGFR 1 – FGFR4, and detected in both humans and mice. These genes encode receptor tyrosine kinases that are approximately 800 amino acids long and contain an extracellular ligand-binding domain with three immunoglobulin-like domains (I, II and III), a transmembrane domain and a divided intracellular tyrosine kinase domain. The immunoglobulin-like domain III is an essential structural site with high ligand-binding specificity.[84] To summarize, previous research has determined 7 major FGFR proteins (FGFRs 1b, 1c, 2b, 2c, 3b, 3c and 4) to be generated from four different FGFR genes, via alternative and specific splicing, with various ligand-binding specificity. This diversity

contributes to the wide spectrum of biological activities induced by these growth factors, and has added majorly to their importance to participate in molecular and cellular processes.[85]

As mentioned before, the paracrine FGFs have a heparin-binding site and interact with their corresponding receptors to induce their biological activity. It has been established that heparin-like molecules are necessary for the stable interaction with FGFRs and their immunoglobulin-like domain III in order to induce proper local signaling.[86] It has been shown that sufficient activation can only be induced when a corresponding co-factor is present, the FGF protein alone binds rather insufficiently to the corresponding receptor and binding occur only very weakly, with insufficient conformational changes of the receptor, hence insufficient signal transduction. It is known that when FGF binding occurs, FGFRs undergo a functional dimerization, receptor transphosphorylation and activation of four key downstream signaling pathways: RAS-RAF-MAPK, PI3K-AKT, STAT and PLCγ.[86, 87]

The FGF proteins known to act paracrine on their neighboring cells are greatly dependent on appropriate receptor activation and signal induction. The last groups that need mentioning are the endocrine FGFs, FGF15/19, FGF21 and FGF23, which are postulated as well to mediate their biological responses in an FGFR-dependent manner.[88] Different from their paracrine siblings, the endocrine FGFs have shown that they bind to their corresponding receptor and heparin/heparan sulphate as a co-factor with very low affinity. The observation of their reduced heparin-binding affinity was postulated to explain that this specifically enables endocrine FGFs to function in an endocrine manner, acting not paracrine on their neighboring

cells but having the ability to participate in signaling processes on different tissue and organ systems.[89]

6.2 Fibroblast Growth Factor 23

Fibroblast Growth Factor 23 (FGF23) is a 30-kDa protein, containing 251 amino acids, with an N-terminal region containing the FGF homology domain and a novel 71-amino acid C terminus. FGF23 can be inactivated when cleaved at the ^{176}R*XX*R^{179} motif, generating biologically inactive N- and C-terminal fragments.[90]

The protein FGF23 acts as a phosphaturic factor and has been identified as a pathogenetic molecule to be involved in three different hypophosphatemic diseases and the inappropriate regulation of vitamin D metabolism, since it has the ability to strongly suppress the 1α-hydroxylase activity in the kidney. Its features make it functionally distinct from other members of this family.[91]

Firstly, FGF23 was identified to act as a phosphatonin in patients with autosomal dominant hypophosphatemic rickets (ADHR), where its mutations stabilize the FGF23 protein. The mutations occur at the ^{176}R*XX*R^{179} site (R176Q, R179W, and R179Q) where they prevent cleavage and inactivation of FGF23 leading to increased circulating levels.[92] Another disease state with inappropriately high levels of FGF23 is X-linked hypophosphataemia (XLH), a disease caused by inactivating mutations of the PHEX gene, the loss of a membrane-bound protease resulting in increased circulating levels of FGF23.[93, 94]

Finally, in tumor-induced osteomalacia (TIO), also called oncogenic osteomalacia, tumors secrete large amounts of FGF23. This disease is an acquired hypophosphatemic disorder with phenotypic similarity to ADHR and XLH. Based on the phenotypic similarity between the three diseases, an unproven model has been postulated. When presumed that PHEX is involved in degrading active full length FGF23 into inactive fragments and increased levels of FGF23 are causing the phosphate wasting in ADHR, XLH and TIO, previous research has shown that in ADHR and XLH a mutation in the FGF23 genes occurs that renders it resistant to PHEX dependent cleavage, in TIO the increased production of FGF23 by the tumor overwhelms the capacity of PHEX to influence the inactivation of FGF23 proteins.[94] Leaving all three diseases stated with the phenotypic outcome of inappropriate inorganic phosphate wasting.[95]

Based on this finding, recent studies have determined FGF23 to be a phosphaturic hormone that is able when not effectively degraded by the PHEX dependent pathway, to inhibit sodium-dependent phosphate uptake in the renal proximal tubule, causing massive phosphate wasting and additionally inhibiting the activation of Vitamin D by the active suppression of 1α-hydroxylase.

Early on FGF23 has been identified to be secreted by osteocytes.[96] Yet its physiological role as an endocrine hormone primarily targets the kidney and parathyroid glands. This biological action plan has led to the definition of the so called kidney-bone axis regulated by FGF23, where it also regulates the bone mineralization via its ability to induce renal phosphate

wasting down, down regulating the Na/Pi co-transporters and causing suppression of renal and intestinal phosphate reabsorption.[97] FGF23 induced hyperphosphaturia has been associated with significant decrease of bone mass and osteomalacia. In summary, FGF23 impact on the kidney derives from the fact that it has a highly regulating role in the control of renal phosphate homeostasis.

As mentioned in the beginning, FGF23 has been associated with an inhibition of 1α-hydroxylase , which is expressed in the proximal convoluted tubule, distal convoluted tubule, the cortical and medullary part of the collecting ducts [98] and mediates the conversion of native vitamin D to calcitriol. The study from Shimana et al.[96] provided proof that FGF23 yet regulates renal expression of Na/Pi2a co-transporter in a Vitamin D Receptor (VDR) independent manner and that calcium is a potent stimulator of FGF23 production via VDR-independent pathway and that via the suppression of the 1α-hydroxylase FGF23 directly impacts the conversion of Vitamin D to calcitriol.[91, 99] FGF23 has his position as a regulator that acts upon feedback to maintain physiological phosphate balance, excess of FGF23 induces hypophosphatemia which mediates the suppression of PTH, on the other hand induces FGF23-mediated low calcitriol levels the stimulation of PTH. FGF23 also acts directly on the parathyroid tissue to inhibit PTH synthesis and secretion. The complexity of the FGF23 mediated regulation of PTH and the feedback loop via the suppression of Vitamin D has been illustrated by Komaba et al, as presented below.[100]

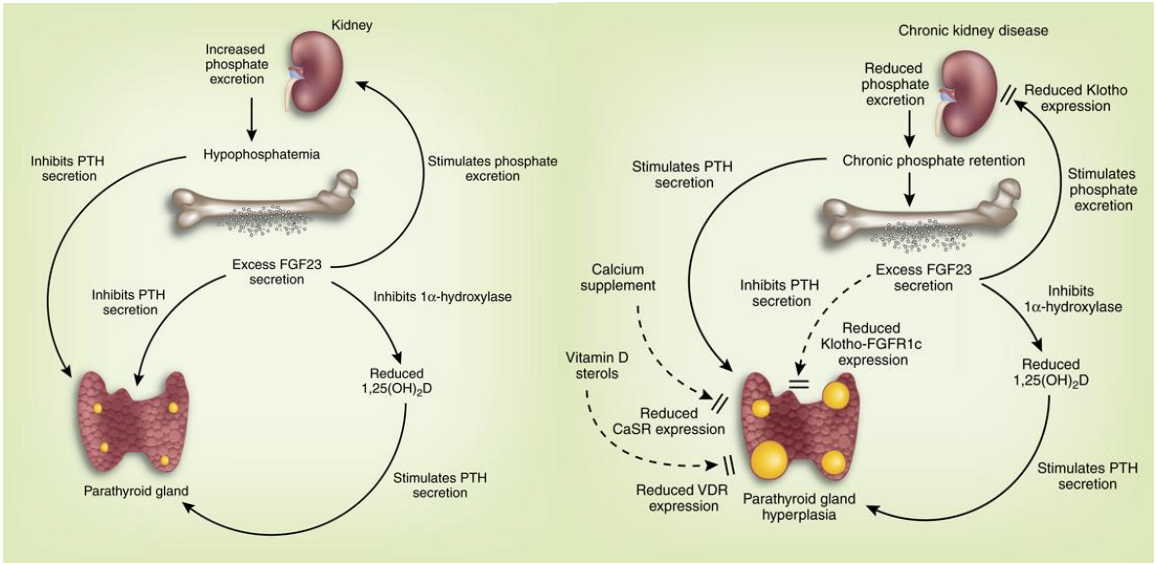

Figure 1: FGF23–parathyroid interaction: implications in chronic kidney disease[100]

6.3 The FGF23 involvement in chronic kidney disease

Since FGF23 has been established as an important hormonal factor to impact on renal physiology, studies have been conducted to clarify its role on CKD. Fliser et al[101]. have determined in the MMKD study that elevated circulating FGF23 levels are also found in patients with advanced CKD. In this setting the elevation of serum FGF23 levels is thought to counteract the retention of phosphate and is known to be accompanied by a rise in PTH and a fall in calcitriol levels.[17, 102]

Along the line of FGF23 counteracting the retention of phosphate to maintain balance and act as a regulator in CKD, Guiterrez et al. [103] have associated increased levels of FGF23 with increased mortality amongst patients on hemodialysis. They determined that FGF23 was an earlier marker for risk of death even before first hyperphosphatemia develops. This study triggered the question of the temporal sequence of events and changes of the involved

parameters and led to further findings that FGF23 in fact precedes the rise of PTH and the drop of calcitriol, positioning it as a primary control marker for CKD progression.[17, 102] Although this has been postulated for some time, only most recent studies could provide proof of the early rise of FGF23 as a potential regulator, leaving the question what is the initial trigger to activate the osteocytes to enhance FGF23 secretion?

6.4 Klotho – Autonomous Autocrine Hormone and Co Factor to FGF23

The Klotho gene encodes a 130-kDa single-pass transmembrane protein with a short cytoplasmic domain, consisting of 10 amino acids.[19] Studies have determined that the short cytoplasmic tail is cleaved by ADAM 10 and 17, once it is translocated from the endosome to the cell membrane, the extracellular domain of Klotho (soluble α-Klotho) is cleaved and released into the blood stream, acting as a paracrine hormone inducing renal signalling.[20] Secreted Klotho inhibits the Na/Pi co-transporter [104] and activates ion channels TRPV5, TRPV6, causing increased cell-surface abundance of the renal epithelial Ca^{2+} channel TRPV5 by modifying its N-linked glycans.[105]

Klotho is expressed predominantly in the kidney, but has been detected as well in the parathyroid glands and the choroid plexus, the function in these tissues is yet to be completely clarified.[19] Imura at al. recently reported that Klotho interacts with the Na/K/ATPase in all of the determined tissues of its expression. Specifically in the choroid plexus it binds to the intracellular domain of the Na/K/ATPase, forming a binding structure that travels to the cell

surface with Klotho. Yet the molecular mechanism for this specific process still lacks exact explanation.

Mice carrying a loss-of-function mutation in the Klotho gene develop a syndrome resembling human aging, including shortened life span, skin atrophy, muscle atrophy, osteoporosis, arteriosclerosis, and pulmonary emphysema. On the other hand, overexpression of the Klotho gene extends the life span and increases resistance to oxidative stress in mice. These observations suggest that the Klotho gene functions as an aging suppressor gene. The shedded domain of Klotho (soluble α-Klotho) is hypothesized to suppress the intercellular signaling of insulin/IGF1 pathway, which partly contributes to its anti-aging properties.[19, 106, 107]

The phenotypic resemblance of Klotho and FGF23 KO mice has led to the assumption that they might influence each other and subsequent to this findings it has been stated that the soluble α-Klotho has the ability to bind canonically to the FGFR 1(IIIc) converting it into a specific receptor for FGF23. This FGF23 receptor shows a strong affinity that is sufficient for interacting with physiological concentrations of FGF23, leading to the hypothesis that the biological activity of FGF23 is regulated by the local distribution of Klotho. The discovery of Klotho as a receptor modulator has added a potential explanation for Klotho as a potential early biomarker in CKD.[108]

One possibility is that Klotho, in addition to being the co receptor for FGF23, has other downstream roles. It needs to be emphasized that the primary site of FGF23 action in the

kidney is postulated to be the proximal renal tubule, yet the primary expression site of Klotho in the kidney is the distal convoluted tubule. The ability of the shedded α-Klotho to act as a proteohormon in a paracrine way contributed to the hypothesis that its influence on phosphate reabsorption and Vitamin D activation is indeed in the proximal tubule.[104]

Additionally to Klothos function as a co-factor to FGF23, it has been associated with the physiological regulation of calcium. This finding was based on Klotho mutant mice that presented significantly elevated levels of calcium, phosphate and active Vitamin D (1.25-OH). The mutant animals developed prematurely and became significantly calcified mainly in the kidney. The setting suggested that the calcification was due to the elevated metabolites that provoke extensive tissue damage that contributed finally to the phenotype of progressive aging. Specifically the excess of active Vitamin D has been associated with advanced and progressive aging, nominating it as a direct factor. Tsujikawa et al.[109] demonstrated that life span of the Klotho mutant mice can be prolonged when the animals are put on a Vitamin D deficient diet. In the physiological process of regulating calcium in organisms, PTH has been established as essential factor in renal patients regarding the control of their mineral ion metabolism. One of the main functions of PTH regarding the kidney is the maintenance of calcium balance in serum, protecting the organism from hypocalcaemia. In relation to Klotho and its contribution to calcium regulation, PTH has soon been tied to Klotho, identifying Klotho as a potential early regulator of PTH secretion. PTH secretion is known to be regulated by a G-coupled protein calcium sensing receptor,[110] the interaction of the Na/K/ATPase Klotho complex has been added to the list of regulators, based on the finding

that in Klotho knockout mice PTH secretion seems to be completely blunted. It remains to be clarified if the action of Klotho on the Na/K/ATPase has a central role or is a secondary effect on PTH via its regulation of the active Vitamin D metabolite.[111]

7 Purpose

The regulatory role of phosphaturic hormones has been intensely studies since the beginning of the 21st century. The research efforts in the topic of FGF23 and Klotho as regulatory factors and early biomarkers have contributed immensely to picture of sHPT development and consequences of chronic renal failure. Yet, in order to establish these molecules as valid for daily clinical practices, such as early screening markers or potential hormonal therapeutics, the picture yet needs to be completed.

The purpose of this dissertation was the analysis of the mineral ion homeostasis in setting of early chronic kidney failure, specifically the temporal course of change regarding the 4 parameters: Vitamin D, PTH, FGF23 and Klotho. As a model for early CKD we initially used autosomal polycystic kidney disease, where we included only patients with preserved kidney function. We examined phosphate homeostasis in the early onset of polycystic kidney disease, studying the different parameters known to be influencing and contributing to development of secondary hyperparathyroidism. Our focus was the expression of FGF23 and Klotho in such

an early onset of the disease, where we searchedfor possible explanations in regards to the clinical setting concerning the ADPKD patients.

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Patients with autosomal dominant polycystic kidney disease have elevated fibroblast growth factor 23 levels and a renal leak of phosphate

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Fibroblast growth factor 23 (FGF23) and parathyroid hormone blood levels rise following progressive loss of renal function. Here we measured parameters of phosphate metabolism in 100 patients with autosomal dominant polycystic kidney disease (ADPKD) in stage 1 or 2 of chronic kidney disease, 20 patients with non-diabetic chronic kidney disease, and 26 with type 2 diabetes. Twenty healthy volunteers served as controls. The mean levels of FGF23 were significantly (4-fold) higher in ADPKD compared to non-diabetic and diabetic patients, and healthy volunteers. Mean serum phosphate levels were significantly lower in ADPKD patients compared to non-diabetic and diabetic patients, and the healthy volunteers. The prevalence of hypophosphatemia was 38, 25, 27, and 5% in ADPKD, non-diabetic and diabetic patients, and healthy volunteers, respectively. The tubular maximum of phosphate reabsorption per glomerular filtration rate was lowest in ADPKD patients with a significantly high positive correlation with serum phosphate levels. Estimated glomerular filtration rates were approximately 100 ml/min per 1.73 m^2 in all groups and parathyroid hormone and vitamin D metabolite levels were in the normal range. Thus, FGF23 was substantially elevated in ADPKD patients compared to other CKD patients matched for glomerular filtration rate, and was associated with increased renal phosphate excretion. The mechanism for this anomaly will require further study.

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KEYWORDS: autosomal dominant polycystic kidney disease; fibroblast growth factor 23; parathyroid hormone

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Autosomal dominant polycystic kidney disease (ADPKD) is a slowly progressive disease, which is caused by mutations in *PKD1* or *PKD2*, the genes encoding for polycystin-1 and -2. The growing cysts gradually replace the functional renal parenchyma and distort the normal architecture of the kidney. Typically, the volume of the kidney increases from normal (150–200 cm^3) in adolescence to 1000 cm^3 in young adults.[1–2] At an age between 20 and 40 years, ~50% of the normal parenchyma is replaced by cysts. However, despite the presence of innumerable cysts in both kidneys, glomerular filtration rate (GFR) remains preserved up to the age of 40 years in most patients, because glomerular hyperfiltration of functioning nephrons compensates for the ongoing loss of functional renal tissue.[3–4]

Fibroblast growth factor 23 (FGF23) is primarily secreted by osteocytes and regulates bone mineralization.[5] In the kidney, FGF23 binds to the canonical FGF receptor in the presence of the cofactor Klotho and suppresses renal tubular phosphate reabsorption and 1α-hydroxylase activity.[6] Hyperphosphaturia, decreased bone mass, and osteomalacia are hallmarks of disorders with excessive FGF23 production and normal renal function, which include autosomal dominant and recessive hypophosphatemic rickets,[7] X-linked hypophosphatemia,[8] and oncogenic osteomalacia.[9] Markedly elevated circulating FGF23 levels are also found in patients with advanced chronic kidney disease (CKD). In such patients, the elevation of serum FGF23 levels is thought to counteract the retention of phosphate and is classically accompanied by a rise in parathyroid hormone (PTH) and a fall in 1,25-dihydroxyvitamin D levels.[10] However, whether the rise in serum FGF23 in patients with CKD precedes or follows the reduction in GFR remains a matter of debate.

The objective of the present investigation was to measure FGF23 serum levels in a cohort of ADPKD patients with CKD stage 1 and 2 and to correlate serum FGF23 levels with parameters of phosphate metabolism, including renal phosphate excretion, serum PTH levels, and levels of vitamin D metabolites. Surprisingly, FGF23 was substantially elevated

in ADPKD patients compared with GFR-matched CKD patients, and was associated with an apparent renal phosphate leak, while PTH and vitamin D metabolite levels remained in the normal range. This suggests that the marked increase of FGF23 in early stage ADPKD represents a previously unrecognized manifestation of ADPKD.

RESULTS

Patient characteristics

Table 1 shows the characteristics of 100 patients with ADPKD (mean age 31 years, 64% male), 20 patients with non-diabetic CKD (NDCKD) (mean age 37 years, 40% male), 26 patients with type 2 diabetes mellitus (DM2) (mean age 57 years, 81% male), and 20 healthy volunteers (mean age 32 years, 60% male). The estimated GFR was similar in all groups (mean estimated GFR 94 ml/min per 1.73 m^2 in ADPKD, 98 ml/min per 1.73 m^2 in NDCKD, 92 ml/min per 1.73 m^2 in patients with DM2, and 99 ml/min per 1.73 m^2 in healthy volunteers). The mean total kidney volume was 967 ± 534 cm^3 in ADPKD patients. ADPKD-related symptoms such as flank pain, past episodes of macrohematuria, and cyst infection were reported in up to 30% of the patients. Chronic glomerulonephritis was present in 18 patients of the NDCKD patients (biopsy proven in 14 patients) and 2 patients had ischemic nephropathy. In patients with DM2, the mean diabetes duration was 8 ± 5 years, the mean glycosylated hemoglobin (HbA1c) level was 8.6 ± 1.0% and the antidiabetic treatment included insulin and oral drugs. The kidneys of the healthy volunteers were normal by ultrasound examination with a mean total kidney volume of 360 ± 80 cm^3. The median urinary excretion of albumin and the prevalence of microalbuminuria were similar in ADPKD and NDCKD patients. None of the patients or volunteers received treatment with vitamin D, vitamin D analogs, calcium supplementation, phosphate binders, or bisphosphonates.

FGF23, serum phosphate, and urinary phosphate reabsorption

The mean c-term FGF23 level in the ADPKD group amounted to 163 relative unit (RU)/ml and was significantly higher than in NDCKD patients (44 RU/ml), patients with DM2 (40 RU/ml), and healthy volunteers (28 RU/ml) (Table 2). The FGF23 levels were similar in NDCKD, DM2, and healthy volunteers groups. ADPKD patients (99%), none of NDCKD patients, 15% of patients with DM2, and none of the healthy volunteers had a c-term FGF23 level >100 RU/ml. The difference between ADPKD patients and the other two patient groups with CKD remained significant independent of the level of albuminuria, estimated GFR, measured creatinine clearance, or the presence of hypertension. The higher c-term FGF23 levels in ADPKD patients were independent of renal function and age (Figure 1).

Intact FGF23 and c-term FGF23 levels were measured simultaneously in a subset of 10 serum samples of ADPKD patients. There was a highly significant correlation between c-term FGF23 levels measured by the Immutopics assay and intact FGF23 levels measured by the Kainos assay ($r^2 = 0.68$, $P = 0.003$) without difference between the assay means (0 ± 39 RU/ml, $P = 0.99$).

Table 1 | Characteristics of patients with ADPKD, DM2, or NDCKD, and of HVs[a]

Characteristic	ADPKD, *N*=100	NDCKD, *N*=20	DM2, *N*=26	HV, *N*=20
Age (years)	31 (6)	37 (17)	57 (10)	32 (5)
Sex, n (%)				
Female	36 (36)	12 (60)	5 (19)	8 (40)
Male	64 (64)	8 (40)	21 (81)	12 (60)
BMI (kg/m^2)	24 (4)	24 (3)	30 (3)	24 (2)
Estimated GFR (ml/min per 1.73 m^2)[b]	94 (18)	98 (18)	92 (14)	99 (12)
Chronic kidney disease classification, n (%)[c]				
Stage 1	53 (53)	13 (65)	14 (54)	—
Stage 2	46 (46)	7 (35)	12 (46)	—
Stage 3	1 (1)	0 (0)	0 (0)	—
Urinary albumin excretion (mg/day)	47 (27–97)	44 (14–264)	10 (5–17)	6 (4–9)
Blood pressure (mm Hg)				
Systolic	131 (16)	131 (8)	131 (11)	123 (7)
Diastolic	83 (11)	84 (5)	85 (10)	74 (6)
Antihypertensive treatment, n (%)				
ACEi/ARB	37 (37)	16 (80)	18 (69)	0 (0)
Thiazide diuretic	9 (9)	1 (5)	10 (38)	0 (0)
Others	7 (7)	0 (0)	5 (19)	0 (0)

Abbreviations: ACEi/ARB, angiotensin-converting-enzyme inhibitor or angiotensin receptor blocker; ADPKD, autosomal dominant polycystic kidney disease ; BMI, body mass index; DM2, type 2 diabetes mellitus; GFR, glomerular filtration rate; HV, healthy volunteer; NDCKD, non-diabetic chronic kidney disease.
[a]Table shows the mean (s.d.), the median (interquartile range), or number of patients (%).
[b]The GFR was estimated by using the Chronic Kidney Disease Epidemiology Collaboration equation.[36]
[c]Chronic kidney disease was classified according to the Kidney Disease Outcomes Quality Initiative of the National Kidney Foundation.[37] Stage 1 denotes GFR rate (ml/min per 1.73 m^2) >90; stage 2, 60–89; and stage 3, 30–59.

Table 2 | Parameters of phosphate metabolism in patients with ADPKD, NDCKD, or DM2, and in HVs

Parameter	ADPKD, *N*=100	NDCKD, *N*=20	DM2, *N*=26	HV, *N*=20	*P*-value
Fibroblast growth factor 23 (RU/ml)	163 (33)	44 (18)	40 (56)	28 (22)	<0.0001
Mean difference from CKD	120†	Reference	−3	−16	—
95% CI	99–140	—	−28 to 21	−43 to 10	—
Serum phosphate (mmol/l)	0.92 (0.17)	1.04 (0.22)	0.96 (0.17)	1.01 (0.12)	0.007
Mean difference from CKD	−0.11†	Reference	−0.08	−0.02	—
95% CI	−0.21 to −0.02	—	−0.18 to 0.04	−0.15 to 0.10	—
TmP/GFR (mmol/l per glomerular filtration)	0.81 (0.18)	0.92	NA	0.89 (0.12)	0.04
Mean difference from CKD	−0.10†	Reference	—	−0.04	—
95% CI	−0.21 to −0.01	—	—	−0.16 to 0.09	—
24-h renal phosphate excretion (mmol per 24 h)	21 (13)	25 (10)	NA	25 (11)	0.2
Mean difference from CKD	−4	Reference	—	1	—
95% CI	−11 to 3			−9 to 10	
Measured creatinine clearance (ml/min per 1.73 m²)	99 (25)	111 (18)	NA	113 (49)	0.1
Mean difference from CKD	−11	Reference	—	2	—
95% CI	−29 to 6	—	—	−20 to 24	—

Abbreviations: ACEi/ARB, angiotensin-converting-enzyme inhibitor or angiotensin receptor blocker; ADPKD, autosomal dominant polycystic kidney disease ; BMI, body mass index; CI, confidence interval; CKD, chronic kidney disease; DM2, type 2 diabetes mellitus; GF, glomerular filtration; GFR, glomerular filtration rate; HV, healthy volunteer; NDCKD, non-diabetic chronic kidney disease; TmP/GFR tubular maximum phosphate reabsorption per GFR.
*Table shows the mean (s.d.).
†Significant for an α level of 0.05, *post hoc* Dunnett's test with NDCKD values as reference.

As FGF23 promotes phosphate excretion by the kidney, we examined phosphate metabolism in detail. Table 2 shows that the mean serum phosphate levels were lowest in ADPKD patients (0.92 mmol/l in ADPKD patients, 1.04 mmol/l in NDCKD patients, 0.96 mmol/l in DM2 patients, and 1.01 mmol/l in healthy volunteers). The mean difference (95% confidence interval) of serum phosphate levels between ADPKD and NDCKD patients was −0.11 (−0.21 to −0.02) mmol/l. The serum levels were similar in NDCKD, DM2, and healthy volunteers groups. Figure 2a depicts the relative frequency distribution per 0.1 mmol/l interval of serum phosphate for ADPKD patients, NDCKD patients, and healthy volunteers. The number of patients with hypophosphatemia as defined by serum phosphate level <0.87 mmol/l was highest in the ADPKD group (38%), compared with NDCKD patients (25%), DM2 patients (27%), and healthy volunteers (5%). Moreover, the tubular maximum of phosphate reabsorption per GFR (TmP/GFR) was lowest in ADPKD patients (0.81 mmol/l glomerular filtrate (GF) in ADPKD patients, 0.92 mmol/l GF in NDCDK patients, and 0.88 mmol/l GF in healthy volunteers). Figure 2b displays the relative frequency distribution per 0.1 mmol/l GF interval of TmP/GFR for ADPKD patients, NDCKD patients, and healthy volunteers, and reveals a strong prevalence of low TmP/GFR values in ADPKD patients compared with NDCKD patients and healthy volunteers. There was a very highly correlation between serum phosphate levels and TmP/GFR in ADPKD patients ($r^2=0.86$, $P<0.0001$), NDCKD patients ($r^2=0.96$, $P<0.0001$), and healthy volunteers ($r^2=0.84$, $P<0.0001$) (Figure 2c), suggesting that hypophosphatemia in all conditions was caused by a renal leak of phosphate. None of the study participants presented with hyperphosphatemia. The mean 24-h urinary phosphate excretion was similar in ADPKD, NDCKD patients, and healthy volunteers (21 mmol in ADPKD patients, 25 mmol in NDCKD patients, and 25 mmol in healthy volunteers), and the significant difference of FGF23 levels between ADPKD and NDCKD patients and healthy volunteers was independent of the daily urinary phosphate excretion (Table 2). These data show that the renal phosphate leak causes a high prevalence of hypophosphatemia in ADPKD patients.

PTH, 25-hydroxyvitamin D, and 1,25-dihydroxyvitamin D levels

To elucidate whether the differences in serum phosphate and TmP/GFR were associated with differences in serum levels of other hormones known to influence phosphate homeostasis, serum intact PTH, 25-hydroxyvitamin D, and 1,25-dihydroxyvitamin D levels were measured in ADPKD patients, NDCKD patients, and healthy volunteers. Mean intact PTH (49 ± 16 ng/ml in ADPKD, 35 ± 12 ng/ml in NDCKD, and 43 ± 16 ng/ml in healthy volunteers), 25-hydroxyvitamin D (20 ± 8 μg/l in ADPKD, 19 ± 10 μg/l in NDCKD, and 25 ± 12 μg/l in healthy volunteers), and 1,25-dihydroxyvitamin D levels (50 ± 15 ng/l in ADPKD, 53 ± 27 ng/l in NDCKD, and 51 ± 10 ng/l in healthy volunteers) remained in the normal range in patients with CKD stage 1 and 2 and healthy volunteers (Figure 3). Thus, the elevation of FGF23 in patients with ADPKD and normal or subnormal GFR appears to be a derangement, which is specific for ADPKD and does not appear to be related to changes in other key hormones that regulate phosphate.

DISCUSSION

This study shows that ADPKD patients with CKD stage 1 and 2 have fourfold elevated levels of serum FGF23, whereas PTH, 25-hydroxyvitamin D, and 1,25-dihydroxyvitamin D levels remained in the normal range. The rise in FGF23 was

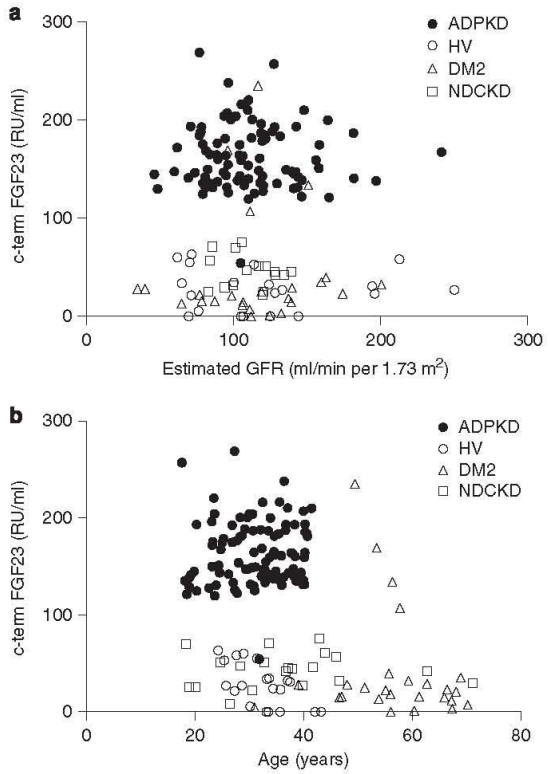

Figure 1 | Fibroblast growth factor 23 versus estimated GFR and age. (**a**) Scatter plot of carboxy-terminal fibroblast growth factor 23 (c-term FGF23) levels versus estimated glomerular filtration rate (eGFR) and (**b**) versus age of 100 patients with autosomal dominant polycystic kidney disease (ADPKD), 20 patients with non-diabetic chronic kidney disease (NDCKD), 26 patients with type 2 diabetes mellitus (DM2), and 20 healthy volunteers (HV). Symbols represent individual patients; bold circles indicate ADPKD patients, open squares NDCKD patients, open triangles DM2, and open circles HV.

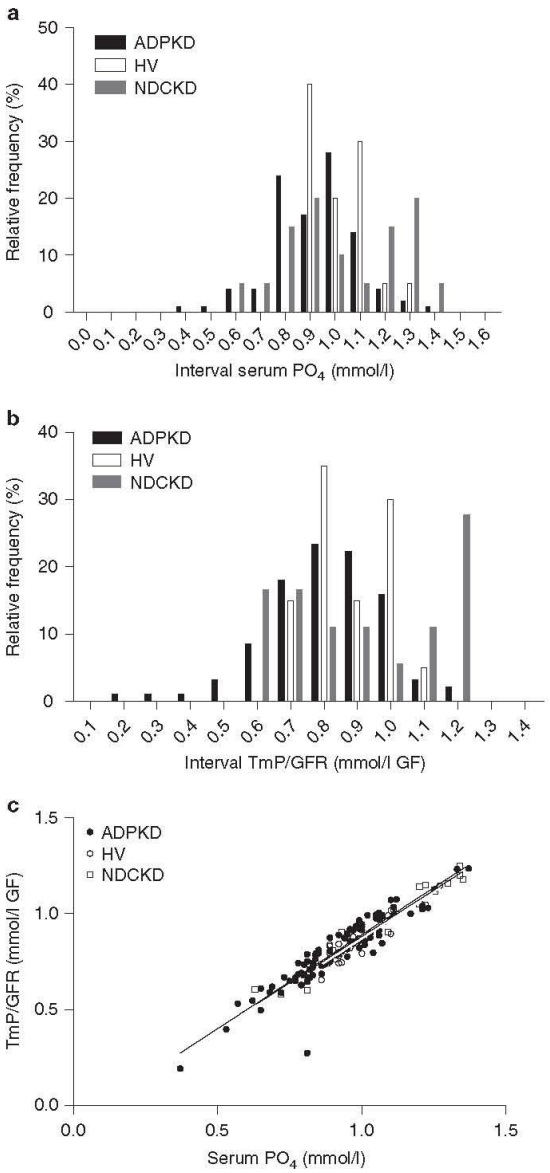

Figure 2 | Serum phosphate and tubular maximum phosphate reabsorption frequency intervals and their correlation. Relative frequency distribution (**a**) per 0.1 mmol/l interval of serum phosphate (PO_4) and (**b**) per 0.1 mmol/ml for tubular maximum phosphate reabsorption per glomerular filtration rate (TmP/GFR) in 100 patients with autosomal dominant polycystic kidney disease (ADPKD), 20 non-diabetic chronic kidney disease patients (NDCKD), and 20 healthy volunteers (HV). (**c**) Correlation between TmP/GFR and serum PO_4 in 100 ADPKD patients ($r^2 = 0.86$, 95% confidence interval (CI) 0.90–1.06, $P < 0.0001$), 20 NDCKD patients ($r^2 = 0.96$, 95% CI 0.85–1.08, $P < 0.0001$), and in 20 HVs ($r^2 = 0.84$, 95% CI 0.76–1.18, $P < 0.0001$). Symbols represent individual patients; bold circles indicate ADPKD patients, open squares NDCKD patients, and open circles HV. Respective lines of linear regression are shown for each group.

accompanied by a renal leak of phosphate, as evidenced by the strong prevalence of low TmP/GFR and by the tight and positive correlation between TmP/GFR and serum phosphate levels. Stated in another way, FGF23 appears to be functionally active, at least at the level of the renal phosphate transport.

Conceptually, FGF23 secretion in CKD is meant to be triggered by phosphate retention as a counteracting mechanism to restore neutral phosphate balance in the face of decreased renal function. In our study, mean estimated GFR and measured creatinine clearance were ~100 ml/min in ADPKD patients. Thus, it appears unlikely that retention of phosphate due to impaired renal function triggered FGF23 secretion. Moreover, in two separate cohorts of CKD stage 1 and 2 patients, FGF23 levels were comprised within the normal range. Therefore, the observation made herein appears as specific for ADPKD.

Previous studies have examined FGF23 levels in patients at early CKD stages. Firstly, it is noteworthy that the serum levels of FGF23 and of phosphate previously reported

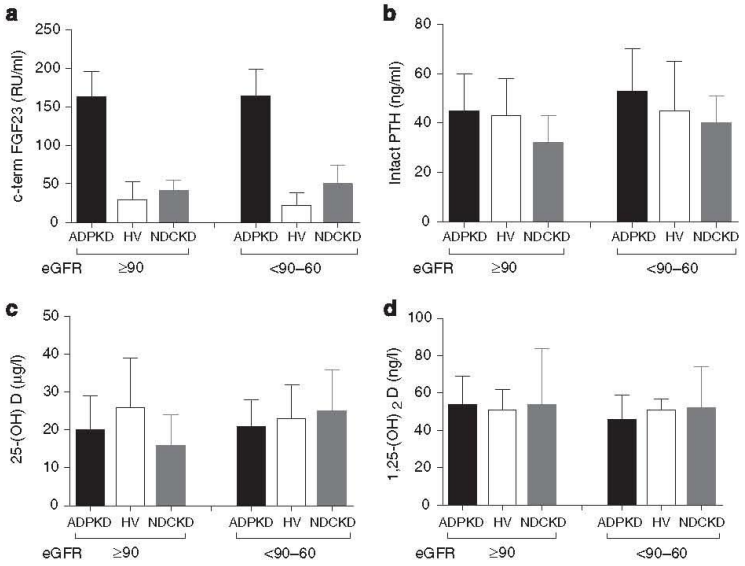

Figure 3 | Fibroblast growth factor 23, parathyroid hormone, and vitamin D metabolites stratified for estimated GFR. (**a**) Carboxy-terminal fibroblast growth factor 23 (c-term FGF23), (**b**) intact parathyroid hormone (iPTH), (**c**) 25-hydroxyvitamin D (25(OH) D), and (**d**) 1,25-dihydroxyvitamin D (1,25$(OH)_2$ D) levels stratified for estimated glomerular filtration rate (eGFR) between 60 and <90, and >90 ml/min per 1.73 m^2 in patients with autosomal dominant polycystic kidney disease (ADPKD), non-diabetic chronic kidney disease (NDCKD) patients, and healthy volunteers (HV).

in patients with CKD 1 and 2[11–17] were similar to those obtained in our patient groups with diabetic and non-diabetic nephropathy. Fliser *et al.*[12] studied FGF23 concentration as a marker for CKD progression and found that the levels of FGF23 steadily increased with decreasing levels of GFR. In that study, CKD patients at stage 1 displayed c-terminal FGF23 levels of 57 RU/ml that increased to 81 RU/ml in CKD stage 2, values that are significantly lower than those measured in our ADPKD cohort. Mean serum phosphate levels were not decreased in CKD stage 1 (1.02 mmol/l) and 2 (1.0 mmol/l) and showed a tendency to increase in CKD stage 3 (1.08 mmol/l). In the study by Gutierrez *et al.*,[11] CKD stage 1 and 2 patients were reported to have a mean c-term FGF23 levels of 86 RU/ml, like in the study of Fliser *et al.*, and serum phosphate values were also reported to be within the normal range with a tendency to rise with decreasing renal function. Notably, both studies reported a parallel increase of intact PTH levels that was already apparent at CKD stage 2, whereas in our study, PTH levels were normal, further indicating an independent mechanism of enhanced FGF23 secretion in ADPKD patients. In both aforementioned studies by Fliser *et al.* and Gutierrez *et al.*, 3 and 16%, respectively, of the study participants were ADPKD patients; however, GFR and FGF23 levels were not reported for this subgroup of patients.

Our findings demonstrate that ADPKD patients display FGF23 levels that are inappropriately high for the degree of severity of renal insufficiency, which mediate a renal phosphate leak and consequently lead to low serum levels of phosphate. Our study suggests that the high levels of FGF23 observed in ADPKD patients are specific to that disease. What could be the explanation for this specificity? FGF23 is also expressed in the liver[18] and ADPKD involves the liver that could have altered the metabolism of FGF23. However, in our cohort of young ADPKD patients, only 5% of them showed cystic liver disease and in most of them only few liver cysts were detected by magnetic resonance imaging. As in all ADPKD patients liver enzymes were in the normal range (data not shown), we considered unlikely that the aforementioned disorder of FGF23 metabolism could originate in the liver.

The Immutopics FGF23 assay detects c-terminal fragments along with its biologically active moiety. It may be speculated that these fragments accumulate in patients with ADPKD due to an altered metabolism, which could account for the elevated c-term FGF23 levels. Therefore, we measured intact FGF23 using the Kainos assay in a subset of ADPKD patients. The difference of the mean between these two assays was very small, indicating that FGF23 fragments did not accumulate in our ADPKD patients and that the elevated levels of c-term FGF23 reflect biologically active FGF23, what is further supported by the observed renal leak of phosphate. Actually, our results are in line with a previous study in which the relationship between different FGF23 assays were tested in normophosphatemic individuals.[19]

It is fair to state that the stimulus driving FGF23 secretion in patients with CKD is incompletely understood. It is tempting to postulate that the synthesis of FGF23 is induced by changes in intracellular phosphate in response, for instance, to

chronic loads in dietary phosphate. Along that line it could be speculated that for unknown reasons our ADPKD patients would chronically ingest more phosphate than the volunteers and CKD patients, and that this higher amount of phosphate would be triggering an increase of FGF23 secretion.[20] However, such was not the case because there was no difference in the 24-h urine excretion rate of phosphate between the three groups. In addition, Isakova *et al.*[21] showed that an acute oral phosphate and calcium load did not change FGF23 levels in normophosphatemic and normocalcemic CKD patients, and it would be surprising that differences in phosphate intake among the four groups could explain a fourfold increase of FGF23 levels in ADPKD patients.

Although renal cysts are the most prominent feature of the disease, ADPKD is a systemic disorder that involves the heart and vessels, liver, pancreas, and lung.[22–24] Interestingly, polycystin-1 is highly expressed in osteoblasts and osteocytes, the main sources for FGF23 production.[25] Targeted disruption of *Pkd1* in mice causes severe defects in bone development, suggesting that polycystin-1 has a major role in the embryonic formation of cartilage and bone.[26–30] Conditional disruption of *Pkd1* in osteoblasts leads to bone loss in mice, suggesting that polycystin-1 regulates bone metabolism, although the mechanism still needs to be determined.[31] It could be hypothesized that polycystin-1 is directly implicated in the regulation of FGF23 production, and that a genetic defect of polycystin-1 as the one occurring in ADPKD is responsible for the increased FGF23 secretion. In contrast to the role of polycystin-1 in bone formation and metabolism in rodents, an ADPKD specific alteration of bone metabolism has so far not been reported in human ADPKD disease. Thus, the clinical consequences of an enhanced FGF23 secretion in ADPKD patients are currently unknown.

In conclusion, the finding of elevated FGF23 levels in ADPKD with normal renal function, normal PTH ,and apparent renal leak of phosphate represents a previously unrecognized manifestation of ADPKD. However, further studies are needed to unmask the mechanism governing this disorder.

METHODS

Study participants and procedures

ADPKD patients enrolled in this study belong to the well-characterized prospective SUISSE ADPKD cohort.[32] Patients with previously known ADPKD or with a positive family history for the disease had been screened for enrolment and the diagnosis of ADPKD was based on ultrasonographic diagnostic criteria.[33] Patients aged 17–40 years with an estimated creatinine clearance $\geqslant$70 ml/min were enrolled. As a comparison to the ADPKD cohort, we studied patients with DM2, aged $>$18 years with an HbA1c level $>$7.5% and an estimated GFR $\geqslant$70 ml/min, patients with NDCKD, aged $>$18 years with an estimated GFR $\geqslant$70 ml/min, and healthy volunteers, aged 17-40 years without history of renal disease, hypertension, or diabetes.

Detailed medical history was obtained from all patients and healthy volunteers, including previous hospitalization and medication. Sitting blood pressure was measured by a nurse after a rest of 5 min. Subjects were declared as having arterial hypertension if systolic blood pressure (SBP) and/or diastolic blood pressure (DBP) were found elevated (SBP $\geqslant$140 mm Hg, DBP $\geqslant$90 mm Hg) or treatment with an antihypertensive drug was recorded. On the morning of the study day and after an overnight fast, a blood sample was drawn. A 24-h urine collection was obtained on the day before the visit to the clinic. A fasting spot urine sample was collected after voiding the first urine of the day before attending the clinics in ADPKD patients and volunteers. Serum and spot urine aliquots were stored at –80°C before analysis. Blood was analyzed for FGF23, PTH, phosphate, creatinine, 25-hydroxyvitamin D, and 1,25-dihydroxyvitamin D. Spot urine was analyzed for phosphate and creatinine. The 24-h urine was analyzed for creatinine and phosphate in the ADPKD, NDCKD, and healthy volunteer group. All ADPKD patients underwent renal magnetic resonance imaging without contrast media. Total kidney volumes were estimated from magnetic resonance imaging sequences as reported previously.[2] The healthy volunteers underwent renal ultrasonography and the kidney volumes were estimated by applying the ellipsoid formula.[34]

All patients were studied at the University Hospital of Zürich, Switzerland, between January 2004 and May 2010. The study was conducted according to the Declaration of Helsinki and the guidelines of Good Clinical Practice. Study approval was obtained from the local ethics committee, and all patients and healthy volunteers gave written, informed consent.

Analytical methods

The levels of carboxy-terminal FGF23 (c-term FGF23, first generation, Immutopics, San Clemente, CA), intact FGF23 (intact FGF23, Kainos Laboratories, Tokyo, Japan), and intact PTH (Biomerica, Newport Beach, CA) were measured by enyme-linked immunosorbent assay according to the manufacturer's protocol. Serum 25-hydroxyvitamin D has been determined using the RIA-kit from Diasorin (Stillwater, MN) and serum 1,25-dihydroxy vitamin D was measured by radioimmunoassay (Immunodiagnostic systems, Fountain Hills, AZ). Phosphate concentrations were measured in serum and urine using standard methods. Creatinine was measured with the use of a modified Jaffe method traceable to an isotope-dilution mass spectroscopy. Albumin was measured on an Integra system (Roche, Rotkreuz, Switzerland), using immunoturbidimetry. Inter- and intra-assay coefficient of variation were $<$5% for the determination of albumin, creatinine, and phosphate, $<$10% for the quantification of 25-hydroxyvitamin D, and $<$13% for 1,25-dihydroxyvitamin D. TmP/GFR in mmol/l of GF was calculated according to the formula of Brodehl.[35] The GFR was estimated by using the Chronic Kidney Disease Epidemiology Collaboration equation.[36] Hypophosphatemia and hyperphosphatemia were defined as serum phosphate levels $<$0.87 and $>$1.45 mmol/l, respectively.

Statistical analysis

Differences among the four groups were compared by one-way analysis of variance. When the difference was significant, statistical comparison of the ADPKD with the NDCKD patients, DM2 patients, and healthy volunteers were performed, using Dunnett's *post hoc* test with the NDCKD as reference group. Differences among these groups in categorical data were compared by the χ^2-test. Univariate Pearson's correlation was used to test for associations between continuous variables. All *P*-values were two sided for the comparison between the groups and values $<$0.05 were considered as statistically significant. Statistical analyses were performed using SAS statistical software version 9.2 (SAS Institute, Cary, NC).

DISCLOSURE
All the authors declared no competing interests.

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Article

Soluble Klotho and Autosomal Dominant Polycystic Kidney Disease

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Summary

Background and objectives Fibroblast growth factor 23 (FGF23) levels are elevated in patients with autosomal dominant polycystic kidney disease (ADPKD) and X-linked hypophosphatemia (XLH), but only the latter is characterized by a renal phosphate wasting phenotype. This study explored potential mechanisms underlying resistance to FGF23 in ADPKD.

Design, setting, participants, & measurements FGF23 and Klotho levels were measured, and renal phosphate transport was evaluated by calculating the ratio of the maximum rate of tubular phosphate reabsorption to GFR (TmP/GFR) in 99 ADPKD patients, 32 CKD patients, 12 XLH patients, and 20 healthy volunteers. ADPKD and CKD patients were classified by estimated GFR (CKD stage 1, ≥90 ml/min per 1.73 m^2; CKD stage 2, 60–89 ml/min per 1.73 m^2).

Results ADPKD patients had 50% higher FGF23 levels than did XLH patients; TmP/GFR was near normal in most ADPKD patients and very low in XLH patients. Serum Klotho levels were lowest in the ADPKD group, whereas the CKD and XLH groups and volunteers had similar levels. ADPKD patients with an apparent renal phosphate leak had two-fold higher Klotho levels than those without. Serum Klotho values correlated inversely with cyst volume and kidney growth.

Conclusions Loss of Klotho might be a consequence of cyst growth and constrain the phosphaturic effect of FGF23 in most patients with ADPKD. Normal serum Klotho levels were associated with normal FGF23 biologic activity in all XLH patients and a minority of ADPKD patients. Loss of Klotho and FGF23 increase appear to exceed and precede the changes that can be explained by loss of GFR in patients with ADPKD.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disorder characterized by bilateral growth of numerous cysts (1,2). Between age 20 and 40 years, the cysts replace approximately 50% of the normal parenchyma (3). In most patients, GFR remains preserved up to age 40 because glomerular hyperfiltration of remaining nephrons compensates for the ongoing loss of functional renal tissue (4,5). ADPKD can easily be detected early in the course of the disease and is an ideal condition to study mineral metabolism at an early stage of CKD because GFR remains stable for a long time, and the disease course is generally not confounded by therapies.

Fibroblast growth factor 23 (FGF23) is a phosphaturic hormone secreted by osteocytes (6,7). Serum levels of this 30-kD peptide were elevated in tumor-induced osteomalacia (8,9), as well as in several genetic diseases, such as autosomal dominant hypophosphatemic rickets secondary to a mutation of the gene that encodes for FGF23 (10,11), autosomal recessive hypophosphatemic rickets secondary to an inactivating mutation of dentin matrix acidic phosphoprotein (*DMP1*) (12,13), and X-linked hypophosphatemia (XLH) secondary to inactivating mutations of *PHEX* (14). As a result of these mutations, FGF23 is less accessible to degradation, and as a consequence, the FGF23 accumulation. leads to severe renal phosphate wasting. In contrast, elevated FGF23 levels in patients with CKD may play an essential role in maintaining normal serum phosphate levels at CKD stages 1 and 2 (15–17). Recently we reported that serum levels of FGF23 are markedly elevated in patients with ADPKD (18). As has been seen with tumor-induced osteomalacia, autosomal dominant hypophosphatemic rickets, autosomal recessive hypophosphatemic rickets, and XLH, this occurred even in the presence of a normal GFR. However, the marked hypophosphatemia typical for tubular disorders is not present in ADPKD, suggesting a resistance to FGF23 in this disease.

The gene *Klotho* encodes for a 130-kD protein with a single transmembrane domain that is mainly expressed in the kidney, the parathyroid glands, and the choroid plexus (19). Once Klotho protein is translocated from the endosome to the cell membrane, the extracellular

domain of Klotho (soluble α-Klotho) may be cleaved and released into the blood stream (20).

FGF23 needs to bind Klotho protein by its C-terminal part to activate the canonical receptor FGFR1c, which leads to reduced membrane expression of the NaPiIIa and NaPiIIc cotransporters as well as downregulation of 1-α hydroxylase (21). Thus, peripheral resistance to FGF23 could be triggered by lower local or circulating levels of Klotho. However, a reliable assay for soluble Klotho has not been available until recently, and data on the expression, function, and regulatory mechanism of soluble α-Klotho are scarce (22).

This study explored potential mechanisms underlying peripheral resistance to FGF23 in ADPKD. We studied the relationship between FGF23, serum Klotho, and renal phosphate handling in patients affected by ADPKD with CKD stage 1 and 2 (ADPKD1-2) and compared it with that in patients with CKD stages 1 and 2 who did not have polycystic kidney disease (CKD1-2), patients with XLH, and healthy volunteers.

Materials and Methods

Study Participants and Procedures

Patients with ADPKD belonged to the well characterized longitudinal observational SUISSE ADPKD cohort (23,24) and were eligible for this study if they had an estimated GFR ≥ than 60 ml/min per 1.73 m^2 according to the CKD Epidemiology Collaboration formula. For comparison, we studied a group of healthy volunteers with age and gender distribution similar to that of the ADPKD cohort, patients with CKD who did not have polycystic kidney disease, and patients with XLH. The ADPKD and CKD patients were classified according to estimated GFR (CKD stage 1, ≥ 90 ml/min per 1.73 m^2; CKD stage 2, 60–89 ml/min per 1.73 m^2). XLH was diagnosed in all patients during childhood when they presented with rickets; diagnosis was based on the presence of clinical and radiographic findings, bowing deformities of the legs, and the laboratory finding of hypophosphatemia due to renal phosphate wasting. Eight of the 12 patients also had a positive family history, and 6 of the 12 underwent corrective orthopedic surgery for their leg deformities. Growth was often impaired despite treatment. Eight of 12 XLH patients were receiving treatment with calcitriol and phosphate (median daily doses of 0.5 μg and 1.25 g, respectively) when the samples were obtained for analysis.

Blood samples were drawn in the morning after an overnight fasting period. The second fasting spot urine samples were collected after the patient voided the first urine of the day before the clinic visit. Blood drawing and spot urine collections were scheduled at the outpatient clinic between 8:00 a.m. and 10:00 a.m. For α-Klotho measurements, aliquots of samples were stored at −80°C before analysis; the exceptions were serum samples from patients with XLH, which were stored at −20°C.

Patients with ADPKD1-2 underwent serial renal magnetic resonance imaging without contrast material every 6 months. Total kidney volumes were estimated from magnetic resonance imaging sequences, as reported elsewhere (3). The healthy volunteers underwent renal ultrasonography, and their kidney volumes were estimated by applying the ellipsoid formula (25).

The study was carried out in accordance with the ethical principles of the Declaration of Helsinki, the Good Clinical Practice guidelines of the International Conference on Harmonization, local regulatory requirements, and local medical ethics committee approval. Patients provided written informed consent.

Analytical Methods

The levels of FGF23 (Immutopics, San Clemente, CA), human soluble α-Klotho (Immuno-Biologic Laboratories Co., Ltd. Japan), and intact parathyroid hormone (Biomerica, Newport Beach, CA) were measured by ELISA according to the manufacturer's protocol.

The novel ELISA method detecting human soluble α-Klotho was developed by first establishing a monoclonal antibody with strong affinity for human α-Klotho protein, recognizing with high selectivity the tertiary protein structure of its extracellular domain. The established protein detection method has been tested by comparing the serum α-Klotho levels of human healthy volunteers with a human case in which the α-Klotho gene carries a mutation that hinders the expression of α-Klotho in the test subject (26). The results of the analysis indicated that the ELISA system can specifically detect and measure the circulating serum α-Klotho levels in humans (22). The ELISA assay was based on the antibodies and substrates designed, used, and described by Yamazaki and colleagues (22). The mean ± SD intra-assay coefficient of variation was 3.6%±1.3%.

In addition to the validation conducted by Yamazaki and colleagues, we performed a sample measurement validation for accuracy at our facility. We performed multiple analyses, measuring human serum Klotho levels from human serum samples that were exposed to different conditions. Samples stored at −80°C with no freeze/thaw cycle for more than 1 year were compared with samples stored at −80°C with one to three freeze/thaw cycles for less than 1 year in order to evaluate thawing stability; freshly collected serum samples were compared with samples kept at room temperature for more than 4 hours in order to evaluate stability at room temperature. The analysis showed that samples with two freeze/thaw cycles remained stable, but further cycles should be avoided. Serum samples kept longer than 4 hours at room temperature showed a slight decrease in reactivity compared with freshly collected serum samples. Finally, we compared samples from various storage conditions (stored at 4°C vs. −20°C) with freshly collected samples. We found no significant differences. Further analyses regarding Klotho expression on the levels of renal tissue could not be performed because we had no access to tissue samples from either study group.

Phosphate and creatinine (isotope dilution mass spectrometry traceable modified Jaffé method) concentrations were measured in serum and urine. The ratio of the maximum rate of tubular phosphate reabsorption to the GFR (TmP/GFR) was calculated as follows:

$$TmP/GFR\ in\ mmol/L = P_P - [U_P \times P_{Crea}/U_{Crea}]$$

where P_P, U_P, P_{Crea}, and U_{Crea} refer to the plasma and urinary concentration of phosphate and creatinine, respectively (27). TmP/GFR allows evaluation of renal phosphate

Table 1. Characteristics of patients according to study group

Characteristic	ADPKD1-2 (*n*=99)	CKD1-2 (*n*=32)	XLH (*n*=12)	Volunteer (*n*=20)
Age (yr)	31±6	40±14	31±11	32±6
Sex, *n* (%)				
female	36 (36)	18 (56)	9 (75)	8 (40)
male	63 (64)	14 (44)	3 (25)	12 (60)
Height (cm)	177±9	171±10	158±9	176±9
Body mass index (kg/m^2)	24±4	26±5	24±4	22±2
eGFR[a] (ml/min per 1.73 m^2)	94±17	94±21	113±18	99±12
BP (mm Hg)				
systolic	131±16	135±12	109±14	123±7
diastolic	83±11	84±8	73±13	75±6
Albumin-to-creatinine ratio (mg/mmol)b	2 (1–3)	3 (1–36)	1 (1–1)	0 (0–1)

Values expressed with a plus/minus sign are the mean ± SD. ADPKD1-2, autosomal dominant polycystic kidney disease with CKD stage 1 and 2; CKD1-2, CKD stage 1 and 2 without polycystic kidney disease; XLH, X-linked hypophosphatemia; eGFR, estimated GFR.
[a]GFR was estimated by using the CKD Epidemiology Collaboration equation (29).
[b]Median (interquartile range).

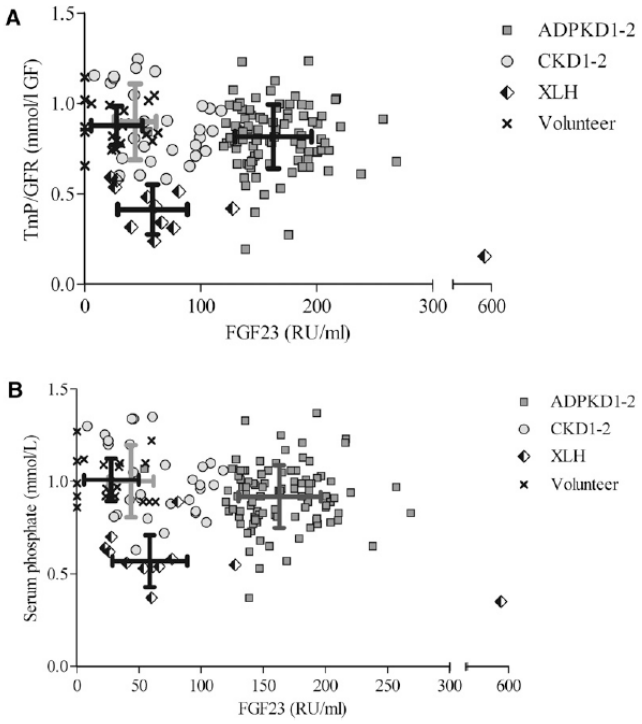

Figure 1. | Fibroblast growth factor 23 (FGF23) versus tubular maximum phosphate reabsorption and serum phosphate. Scatter plots of FGF23 values versus (A) ratio of the maximum rate of tubular phosphate reabsorption to the GFR (TmP/GFR) and (B) serum phosphate of 99 patients with autosomal dominant polycystic kidney disease and CKD stage 1 and 2 (ADPKD1-2), 32 patients with CKD stage 1 and 2 not affected by polycystic kidney disease (CKD1-2), 12 patients with X-linked hypophosphatemia (XLH), and 20 healthy volunteers. Symbols represent individual patients, and lines indicate SDs.

transport and is called the theoretical renal phosphate threshold. This value corresponds to the theoretical lower limit of plasma phosphate below which all filtered phosphate would be absorbed (normal range, 0.80–1.35 mmol/L). TmP/GFR is independent of dietary phosphorus intake, tissue release of phosphorus, and GFR (28). Renal phosphate leak was defined as TmP/GFR < 0.6 mmol/L. The GFR was estimated by using the CKD Epidemiology Collaboration equation (29).

Statistical Analyses

A general linear model approach was applied to calculate least-square means and simultaneous 95% confidence intervals. *P* values were adjusted for multiple comparisons by applying the Dunnett *post hoc* test. Univariate Pearson correlation was used to test for association between continuous variables. Statistical analyses were performed using SAS statistical software, version 9.2 (SAS Institute Inc., Cary, NC).

Results

Study Participants

A total of 99 patients ADPKD1-2, 32 patients with CKD1-2, 12 patients with XLH, and 20 healthy volunteers were enrolled in the study at the University Hospital Zurich between January 2004 and May 2011. Most patients were in their third or fourth decade of life and had an estimated GFR of 80–120 mL/min per 1.73 m^2 (Table 1). Causes of nephropathies in patients with CKD1-2 were IgA nephropathy (nine patients [eight biopsy proven]), Alport or thin basement disease (three patients [two biopsy proven]), minimal change nephropathy (three patients [all biopsy proven]), lupus nephritis (three patients [all biopsy proven]), focal segmental glomerulosclerosis (two patients [all biopsy proven]), other chronic glomerulonephritis (six patients [four biopsy proven]), interstitial nephritis (one patient [biopsy proven]), and hypertensive nephropathy (three patients [none biopsy proven]). As expected, patients with CKD1-2 had higher urinary albumin excretion, and patients with XLH were predominantly female and had lower height than the other groups. The kidneys of the healthy volunteers were normal per ultrasonography, with a mean total kidney volume of 360 cm^3. None of the ADPKD1-2 patients, CKD1-2 patients, or volunteers received nutritional or activated vitamin D metabolites, calcium supplementation, phosphate binders, or bisphosphonates, but most of the patients with XLH were receiving treatment with calcitriol and phosphate supplements (for details, see the Methods section).

Resistance to the Phosphaturic Effect of FGF23 in Polycystic Kidneys

The FGF23 levels were highest among patients with ADPKD1-2, with values approximately 100% higher than those observed in patients with CKD1-2 and 50% higher than those observed in patients with XLH. FGF23 is thought to promote renal phosphate excretion by inhibiting tubular phosphate reabsorption. Thus, we would expect to observe lower TmP/GFR values and serum phosphate levels among patients with ADPKD1-2 than among those with CKD1-2 and those with XLH. However, TmP/GFR values were

Table 2. Phosphate metabolism variables according to study group

Variable	ADPKD1-2 (*n*=99)	CKD1-2 (*n*=32)	XLH (*n*=12)	Volunteer (*n*=20)
Klotho (pg/ml)	719±503	1037±839	1187±569	1200±385
mean difference from ADPKD (95% CI)	Reference	318 (34 to 602)[a]	468 (42 to 895)[a]	481 (140 to 824)[a]
FGF23 (RU/ml)	163±33	67±36	99±143	28±22
mean difference from ADPKD (95% CI)	Reference	−96 (−120 to −72)[a]	−64 (−101 to −28)[a]	−136 (165 to −107)[a]
FGF23-to-Klotho ratio (RU/pg)	0.33±0.25	0.09±0.07	0.08±0.11	0.02±0.02
mean difference from ADPKD (95% CI)	Reference	−0.23 (−0.33 to -0.14)[a]	−0.24 (−0.39 to −0.10)[a]	−0.30 (−0.41 to −0.18)[a]
Parathyroid hormone (ng/ml)	49±16	43±23	57±20	43±16
mean difference from ADPKD (95% CI)	Reference	−6 (−15 to 2)	8 (−5 to 20)	−6 (−16 to 5)
Serum phosphate (mmol/L)	0.92±0.17	1.01±0.19	0.57±0.14	1.01±0.12
mean difference from ADPKD (95% CI)	Reference	0.08 (0.00 to 0.16)[a]	−0.35 (−0.47 to −0.23)[a]	0.09 (−0.01 to 0.18)
TmP/GFR (mmol/L)	0.81±0.18	0.89±0.20	0.41±0.13	0.89±0.12
mean difference from ADPKD (95% CI)	Reference	0.07 (−0.02 to 0.16)	−0.40 (−0.53 to −0.28)[a]	0.07 (−0.03 to 0.17)

Values expressed with a plus/minus sign are the least-square mean ± SD. ADPKD1-2, autosomal dominant polycystic kidney disease with CKD stage 1 and 2; CKD1-2, CKD stage 1 and 2 without polycystic kidney disease; XLH, X-linked hypophosphatemia; CI, confidence interval; FGF23, fibroblast growth factor 23; TmP/GFR, ratio of the maximum rate of tubular phosphate reabsorption to the GFR.
[a]Significant at an α level of 0.05, *post hoc* Dunnett test with ADPKD values as reference.

generally not decreased in patients with ADPKD1-2; they were similar to those found in patients with CKD1-2 and much higher than the values found in patients with XLH (Figure 1A). Notably, TmP/GFR values were inversely (negatively) correlated with FGF23 levels in XLH ($r=-0.63$; $P=0.03$), whereas no correlation could be detected for ADPKD1-2 or CKD1-2. Furthermore, Figure 1B demonstrates that patients with ADPKD1-2 had higher serum phosphate levels than those with XLH and only modestly reduced serum phosphate levels (mean difference, 0.07 mmol/L) compared with CKD1-2 patients and healthy volunteers. Thus, in patients with ADPKD1-2, although serum FGF23 levels were higher than in XLH patients, both the renal threshold of phosphate and serum phosphate levels were higher in polycystic patients, suggesting that the biologic activity of FGF23 was impaired in patients with ADPKD1-2.

Disturbance of the Hormonal Network Regulating Phosphate Metabolism in ADPKD

Patients with ADPKD1-2 had the lowest mean serum level of Klotho, the highest mean serum level of FGF23, and by far the highest serum FGF23-to-Klotho ratio, whereas patients with XLH had the lowest serum phosphate levels and the lowest value for TmP/GFR (Table 2). Parathyroid hormone levels were similar in all groups. Klotho levels were independent of GFR and age (Figure 2) in all groups. Figure 3 summarizes, for ADPKD1-2 (Figure 3A) and CKD1-2 patients (Figure 3B), the individual values of serum Klotho, FGF23 and parathyroid hormone values plotted as a function of GFR over the range of 60–130 mL/min per 1.73 m^2; no correlation was observed. However, it is obvious from the graphs that patients with ADPKD1-2 had lower Klotho levels and higher FGF23 levels than CKD1-2 patients over the whole range of GFR. We also conducted a subset analysis measuring serum Klotho levels in six ADPKD patients with CKD stage 3. These results showed mean ± SD serum levels of 299.0±77.4 pg/ml. This finding concurs with the initial hypothesis that Klotho levels decrease as renal damage progresses.

Low Soluble Klotho Levels Can Be Associated with Low FGF23 Activity in ADPKD

The data were stratified for a functional renal leak of phosphate (TmP/GFR ≥ 0.6 or < 0.6 mmol/L) (Table 3): Thirteen percent of ADPKD1-2 patients, 9% of CKD1-2 patients, 100% of XLH patients, and none of the volunteers had an apparent renal leak of phosphate. Strikingly, among the patients with an apparent renal leak of phosphate, serum levels of Klotho, FGF23, and parathyroid hormone were similar between the groups. On the other hand, among patients without renal leak of phosphate, ADPKD1-2 patients had significantly lower serum Klotho levels, higher serum FGF23 levels, and higher serum FGF23-to-Klotho ratios than CKD1-2 patients or healthy volunteers. In addition, serum Klotho levels were lower in ADPKD1-2 patients without renal phosphate leak than in those with renal leak (659 pg/ml in ADPKD1-2 patients without leak and 1105 pg/ml in ADPKD1-2 patients with leak; mean difference, 446 pg/ml [95% confidence interval,

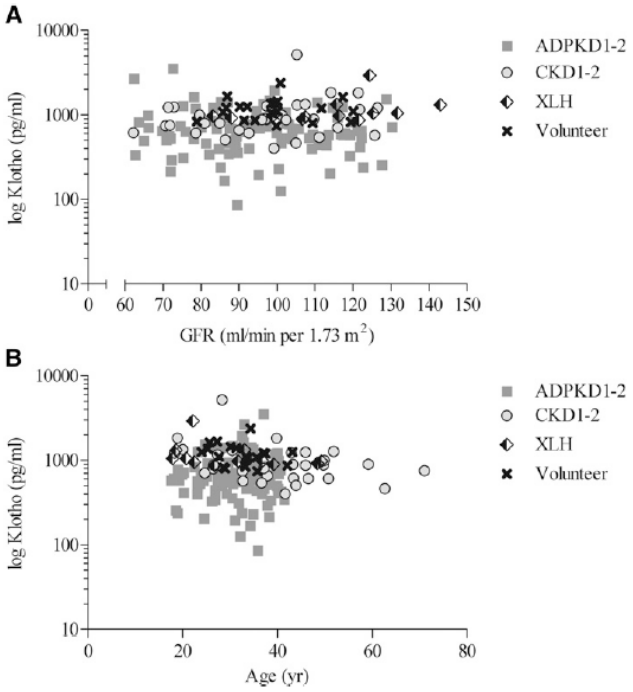

Figure 2. | Klotho versus GFR and age. Scatter plots of values of serum α-Klotho versus (A) estimated GFR and (B) age of 99 patients with autosomal dominant polycystic kidney disease and CKD stage 1 and 2 (ADPKD1-2), 32 patients with CKD stage 1 and 2 not affected by polycystic kidney disease (CKD1-2), 12 patients with X-linked hypophosphatemia (XLH), and 20 healthy volunteers. Symbols represent individual patients.

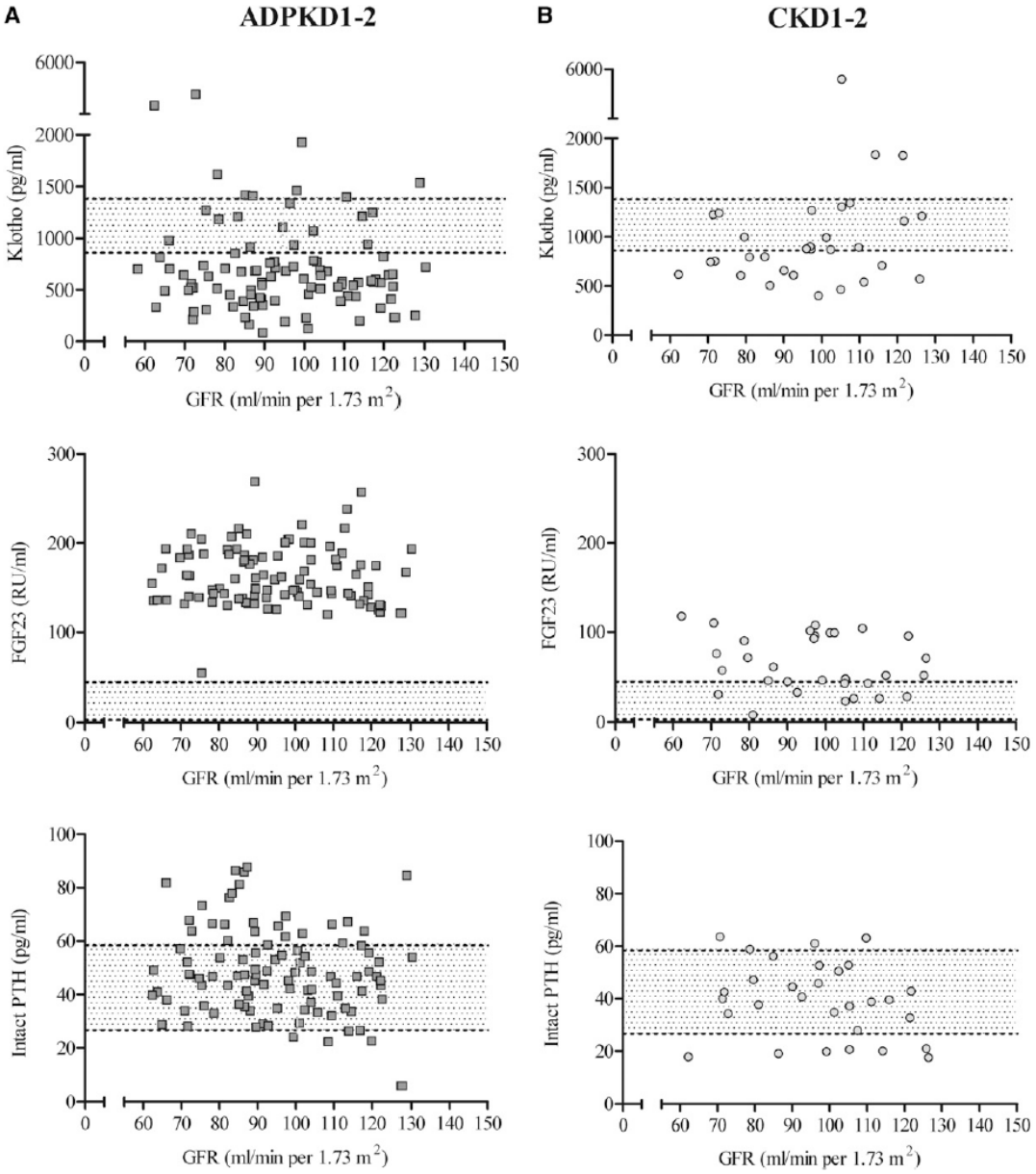

Figure 3. | Klotho, fibroblast growth factor 23 (FGF23), and parathyroid hormone versus estimated GFR. Serum α-Klotho, FGF23, and intact parathyroid hormone (PTH) values versus estimated GFR in (A) 99 patients with autosomal dominant polycystic kidney disease and CKD stage 1 and 2 (ADPKD1-2) and (B) 32 patients with CKD stage 1 and 2 not affected by polycystic kidney disease (CKD1-2). Symbols represent individual patients, and dotted lines represent 25% and 75% quartiles of controls.

161–731 pg/ml]). Age, GFR, gender distribution, and frequencies of angiotensin-converting enzyme inhibitor and angiotensin-receptor blocker use were similar in ADPKD1-2 patients with and in those without renal phosphate leak (data not shown).

ADPKD Phenotype and Klotho

Patients with ADPKD1-2 had a mean ± SD total kidney volume of 970±533 cm^3 and a mean total cyst volume of 561±426 cm^3. The annual mean absolute and percentage kidney growth rates were 78±98 cm^3 and 6.3%±6.9% per year, respectively. The annual mean absolute and percentage cyst growth rates were 98±91 cm^3 and 23%±24% per year, respectively, for a median (interquartile range) follow-up of 36 (25–38) months.

Figure 4 shows that serum Klotho values were inversely correlated with total cyst volume ($r=-0.24$; $P<0.05$) and with annual total kidney growth, both absolute ($r=-0.29$;

Table 3. Phosphate metabolism variables in the study groups, according to presence of renal phosphate leak

Variable	No Apparent Renal Phosphate Leak (TmP/GFR ≥ 0.6 mmol/L)				Apparent Renal Phosphate Leak (TmP/GFR < 0.6 mmol/L)			
	ADPKD1-2 (*n*=86)	CKD1-2 (*n*=28)	Volunteer (*n*=20)	*P* Value	ADPKD1-2 (*n*=13)	CKD1-2 (*n*=4)	XLH (*n*=12)	*P* Value
TmP/GFR (mmol/L)	0.84	0.90	0.88		0.45	0.58	0.41	
Klotho (pg/ml)	659	1016[a]	1201[a]	<0.0001	1105	1185	1187	0.9
mean difference from ADPKD (95% CI)	Reference	357 (80 to 634)	542 (226 to 857)		Reference	80 (−732 to 891)	82 (−486 to 651)	
FGF23 (RU/ml)	165	68[a]	28[a]	<0.0001	152	59	99	0.2
mean difference from ADPKD (95% CI)	Reference	−97 (−113 to −80)	−137 (−156 to −119)		Reference	−93 (−221 to 34)	−53 (−143 to 36)	
FGF23-to-Klotho ratio	0.34	0.09[a]	0.02[a]	<0.0001	0.20	0.06	0.08	0.05
mean difference from ADPKD (95% CI)	Reference	−0.25 (−0.35 to −0.15)	−0.32 (−0.44 to −0.20)		Reference	−0.12 (−0.24 to 0.00)	−0.14 (−0.32 to 0.03)	
Parathyroid hormone (ng/ml)	49	43	43	0.2	46	38	57	0.2
mean difference from ADPKD (95% CI)	Reference	−6.0 (−14.9 to 3.0)	−6.0 (−16.0 to 4.0)		Reference	−8 (−32 to 16)	10 (−6 to 27)	

Values expressed with a plus/minus sign are the least-square mean. TMP/GFR, ratio of the maximum rate of tubular phosphate reabsorption to the GFR; ADPKD1-2, autosomal dominant polycystic kidney disease with CKD stage 1 and 2; CKD1-2, CKD stage 1 and 2 without polycystic kidney disease; XLH, X-linked hypophosphatemia; CI, confidence interval; FGF23, fibroblast growth factor 23.

[a]Significant at an α level of 0.05, *post hoc* Dunnett test with ADPKD1-2 values as reference.

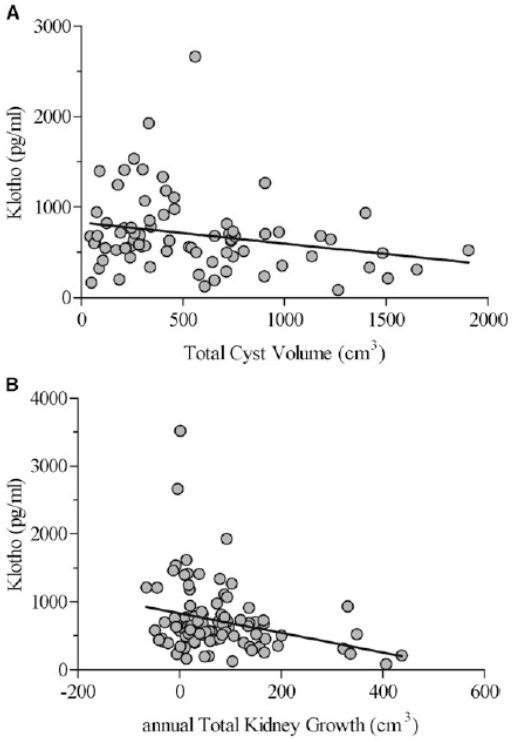

Figure 4. | Klotho versus total cyst volume and annual kidney growth. Serum α-Klotho values versus (A) total cyst volume and (B) annual kidney growth (median follow-up, 3 years) in 99 patients with autosomal dominant polycystic kidney disease and CKD 1 and 2 (ADPKD1-2). Symbols represent individual patients, and linear regression lines are drawn.

$P<0.01$ [shown]) and percentage ($r=-0.27$; $P<0.01$ [not shown]). FGF23 values were not correlated with kidney volume or growth, whereas the FGF23-to-Klotho ratios were positively correlated with total cyst volume ($r=0.22$; $P<0.05$) as well as with absolute ($r=0.39$; $P<0.0001$) and percentage ($r=0.28$; $P<0.01$) kidney growth.

Discussion

Our study reveals a reduced biologic activity of FGF23 in patients with ADPKD1-2 that is characterized by low renal phosphate excretion in the face of excessive FGF23 levels. This finding was accompanied by relatively low serum Klotho levels, suggesting that the resistance to the phosphaturic effect of FGF23 could be mediated by a reduction of Klotho.

FGF23 is secreted by osteocytes and acts on the kidney to promote phosphate excretion into the urine. Higher serum levels of FGF23 have been described in several genetic diseases associated with high urinary phosphate excretion and hypophosphatemia, including patients with XLH. Our XLH patients displayed the characteristic features of renal phosphate wasting ("phosphate diabetes"; TmP/GFR was half that found in healthy volunteers), hypophosphatemia, high FGF23 serum levels (four-fold higher than the levels found in volunteers), short stature, and rickets in childhood; most of them still had impaired bone mineralization in adulthood despite treatment. In patients with XLH we also observed a positive relationship between FGF23 and phosphate wasting (inverse with TmP/GFR), whereas these associations were not present in patients with ADPKD1-2. Furthermore, patients with ADPKD1-2 had even higher FGF23 levels than patients with XLH, but the former did not exhibit renal phosphate wasting. These findings suggest a potential renal resistance to FGF23.

Studies in Klotho-deficient and FGF23-deficient mice have revealed that cofactor binding such as Klotho is essential to initiate FGFR1c activation and thus for FGF23 biologic activity (30). Even extremely high serum FGF23 levels in mice lacking Klotho do not cause a phosphate wasting phenotype, illustrating that the absence of Klotho confers resistance to FGF23 (31). Our data concur with the concept that Klotho is required in humans to permit FGF23 to accomplish its biologic activity. Excessive FGF23 secretion was seen in all patients with ADPKD1-2, but none of these patients had a phosphate wasting phenotype and only 13% had a low TmP/GFR. Remarkably, ADPKD1-2 patients with an elevated renal phosphate excretion had normal serum Klotho levels. However, the majority of patients with ADPKD1-2 had low serum Klotho levels, and we infer that this prevented phosphate wasting. Therefore, we conclude that in this particular inherited kidney disease associated with excessive FGF23 secretion, the loss of Klotho may have contributed to FGF23 resistance.

The mechanisms by which kidney disease decreases Klotho levels remain unknown. Klotho is expressed in tubular epithelial cell of the kidney, with predominant expression in the distal convoluted tubule (19,32). Likewise, cysts evolve from tubular epithelial cells of all parts of the nephrons and may start predominantly in the thick ascending loops of Henle, distal tubules, and collecting ducts in the corticomedullary region and outer medulla (32–34). Intriguingly, serum Klotho levels and cyst volume were inversely correlated with each other. Because we had no access to additional renal tissue samples to measure the level of molecular expression of Klotho as a transmembrane molecule in our patients, we could not clarify whether the level of transmembrane expression of Klotho is specifically reduced in patients with ADPKD. The extracellular domain of Klotho is shed on the cell surface after being cleaved by membrane-anchored proteases (ADAM 10 and 17) and is detected in the blood and cerebrospinal fluid in mice and humans (35,36). Serum (and urine) Klotho levels correlated with Klotho expression in the kidney of Klotho knock-in and knock-out mice, indicating that circulating Klotho may represent renal Klotho expression (37). This shedded soluble Klotho molecule acts as a hormone on the renal proximal tubule, where it has recently been postulated to pass through the basolateral membrane and to migrate through the cytosol; it ultimately reaches the brush-border membrane, where it causes endocytosis of the Na-Pi cotransporters (32). Thus, the low production of Klotho might be related to cyst-induced damage and be responsible, at least in part, for the absence of renal leak of phosphate despite the high levels of FGF23.

Our study results must be interpreted in the context of the study design: We had no access to kidney tissue. It remains speculative to declare at this current stage of

knowledge that soluble Klotho levels mirror kidney Klotho expression in the kidney. Our cross-sectional study was also not designed to elucidate the sequential order of the observed disturbance of the FGF23 and Klotho secretion, and the mechanism causing excessive FGF23 secretion in ADPKD is still not well understood. We speculate that excessive FGF23 secretion and loss of Klotho are two independent processes in which the bone is the origin of the FGF23 production and the loss of Klotho in the polycystic kidney constrains the phosphaturic effect of FGF23.

In conclusion, the renal pattern of ADPKD, classically characterized by cyst development and growth, appears to include, in the early course of the disease, decreased Klotho availability constraining FGF23 activity. Loss of Klotho and the concomitant increase in FGF23 appear to exceed and precede changes mediated by the loss of GFR in patients with ADPKD and could be regarded as early markers of the renal disease in ADPKD.

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Disclosures

None.

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Loss of soluble Klotho precedes FGF23 and PTH rise in chronic kidney disease

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Abstract

Background/Objectives: Peripheral resistance to the actions of FGF23 is known to occur in renal insufficiency, due to down-regulation of expression of Klotho at the level of the parathyroid gland and the kidney. To test whether serum levels of soluble alpha-Klotho reflect this phenomenon, the newly available ELISA for Klotho was applied to measure serum levels in patients with chronic kidney disease (CKD) stages 1 to 5.

Design/Setting/Participants/Measurements: Klotho, fibroblast growth factor 23 (FGF23), parathyroid hormone (PTH), 1,25-dihydroxy vitamin D3 (1,25D) and other parameters of mineral metabolism were measured in serum of 87 patients with various nephropathies. Locally weighted scatter plot smoothing function of these parameters were plotted vs. estimated glomerular filtration rate (eGFR) to illustrate the sequence of events, and appropriate regression models were fitted to estimate the respective changes per each 1ml/min eGFR.

Results: With progressing CKD, Klotho linearly decreased whereas both FGF23 and PTH showed a plateau at early CKD stages and then a curvilinear increase. The adjusted mean Klotho change was -5.7 pg/ml (95%CI -4.1 to -7.5 pg/ml, $P<0.0001$) for each -1 ml/min eGFR change. FGF23 departed from the plateau at an eGFR of 48.6 ml/min (95%CI 41.0 to 56.1 ml/min) whereas PTH departed at an eGFR of 35.3 ml/min (95%CI 21.7 to 50.0 ml/min).

Conclusion: The sequence “low Klotho first - high FGF23 second” observed in CKD suggests a cause-and-effect relationship; serum level of soluble alpha-Klotho might be currently the most sensitive biomarker of renal damage and of CKD progression.

Introduction

Genetic Klotho deficiency in rodents mimics the features of chronic renal failure with accelerated atherosclerosis, osteopenia, soft tissue calcifications, pulmonary emphysema, and altered glucose metabolism.[19] Klotho, expressed in the kidney, parathyroid glands and the choroid plexus[19] is a single transmembrane protein whose extracellular domain is cleaved by the α-secretases ADAM 10 and 17[112] to generate large amounts of soluble α-Klotho into blood, urine, and cerebrospinal fluid.[104, 113] Klotho activates ion channels TRPV5 and 6 in the nephron and the intestine[105, 114] and regulates the sodium-phosphate co-transporter type-2a (NaPiIIa) independently of fibroblast growth factor 23 (FGF23).[104]

Klotho acts as an essential co-factor for FGF23, a phosphaturic hormone produced by osteocytes: it forms a ternary complex with the fibroblast growth factor receptor 1c (FGFR1c), therewith increasing its affinity for FGF23.[115] Thus, FGF23 and Klotho synergize to regulate phosphate homeostasis[116] by promoting renal phosphate excretion: they do so via reduction of the number of NaPiIIa and NaPiIIc phosphate co-transporters in the proximal tubule and reduction of intestinal phosphate absorption, the latter following a decreased synthesis of 1,25-dihydroxy vitamin D_3 (1,25D).[102]

Rodent studies indicate that soluble α-Klotho levels in urine and blood are highly correlated with renal Klotho expression. Studies in patients with chronic kidney disease (CKD) or acute kidney injury indicate a decrease of Klotho expression with decreasing GFR;[117] however, these studies encompass a limited number of patients and Klotho levels were only measured semi-quantitatively by western blots performed in concentrated urine samples.

It has been shown that the curvilinear slope of FGF23 versus estimated glomerular filtration rate (eGFR) ascends earlier than that of parathyroid hormone (PTH) as renal function declines.[17, 118] However, whether the fall in serum Klotho precedes or follows the rise in FGF23 in patients with CKD remains a matter of debate because a reliable assay for soluble Klotho has not been available until recently, and data on the expression, function, and regulatory mechanisms of soluble α-Klotho are scarce.[119]

Our cross-sectional study is the first systematic determination of serum levels of Klotho, FGF23, PTH, 1,25D and other parameters of mineral metabolism performed in a cohort of patients with chronic renal insufficiency at CKD stages 1 to 5D. We were looking for the sequence of the changes in the mentioned parameters over the entire range of CKD stages; the question is relevant as Klotho or FGF23 may turn out to be the most sensitive markers of

early kidney disease, of its progression as well as of its prognosis, besides the fact that both might become future therapeutic targets.[22]

Methods

Study participants and procedures

Eighty-seven patients at different stages of chronic kidney disease (stages 1 to 5D) without polycystic kidney disease or previous kidney transplantation, aged 18-84 years were enrolled in the study. Twenty healthy volunteers, aged 17-40 years without history of renal disease served as control group.

Sitting blood pressure was measured by a nurse, a blood sample was drawn and a spot urine sample (2^{nd} fasting morning urine, after voiding the 1^{st} urine of the day prior to the visit to the clinics) was collected between 8 a.m. and 10 a.m.. The serum and urine aliquots were stored at -80°C. Blood was analyzed for Klotho, FGF23, PTH, phosphate, ionized calcium, creatinine, 25-hydroxyvitamin D (25D) and 1,25D. Spot urine was analyzed for phosphate and creatinine.

The study was conducted according to the Declaration of Helsinki and the guidelines of Good Clinical Practice (GCP) and was approved by the local Ethics Committee. Patients and healthy volunteers gave written, informed consent.

Analytical methods

Soluble alpha Klotho has been measured in serum, a novel ELISA method detecting human soluble α-Klotho has been developed first by establishing a monoclonal antibody with strong affinity for human α-Klotho protein, recognizing with high selectivity the tertiary protein structure of its extracellular domain. The established protein detection method has been subsequently tested comparing the serum α-Klotho levels of human healthy volunteers with a human case where the α-Klotho gene carries a mutation that hinders the expression of α-Klotho in the test subject. The results of the analysis indicated that the ELISA system can specifically detect and measure the circulating serum α-Klotho levels in humans.[119] The intra-assay coefficient of variation was 3.1% (Immuno-Biological Laboratories Co., Ltd. Japan).

The levels of carboxy-terminal FGF23 (2^{nd} generation, Immutopics Inc., San Clemente CA, USA) and intact PTH (Biomerica Inc., Newport Beach CA, USA) were measured in serum by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol.

Serum 25D has been determined using the RIA-kit from Diasorin (Stillwater, MN, USA), and serum 1,25D by radioimmunoassay (immunodiagnostic systems, Fountain Hills, AZ, USA). Phosphate concentrations were measured in serum and urine using standard methods. Creatinine in serum and urine was assayed by the IDMS traceable modified Jaffé

method. The glomerular filtration rate was estimated by using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. The tubular maximum phosphate reabsorption per glomerular filtration rate (TmP/GFR) in mmol/l of glomerular filtrate (GF) was calculated according to the formula of Brodehl.[120]

Statistical Analysis

LOWESS (locally weighted scatter plot smoothing) function of Klotho, 1,25D, FGF23 and PTH versus eGFR in patients with CKD were fitted by the default function of the STATA version 11.2 software (STAT Corp., College Station, TX) with a bandwidth of 0.8. Based on the LOWESS shape, a linear model was fitted for Klotho and 1,25D and the crude mean change for each 1 ml/min/1.73 m^2 estimated eGFR. By fitting an *a priori* model containing the binomial covariates vitamin D supplementation, 1,25D treatment, calcium administration (calcium acetate, calcium supplement), calcium-free phosphate binder (sevelamer, lanthanum), the respective mean estimated changes of Klotho and 1,25D were adjusted for these concomitant medications that potentially influence the rate change. The association between FGF23 and PTH with eGFR was not linear and thus we fitted a segmented model consisting of smoothly fitted plateau and quadratic function to estimate the departure point of the curve from the plateau.

Differences among the CKD stages and healthy volunteers were compared by one-way analysis of variance. When the difference was significant, statistical comparisons were performed by using Dunnett's *post hoc* test with the health volunteers as a reference group. Univariate Pearson's correlation was used to test for associations between continuous variables. All P values were two-sided for the comparison between the groups and values below 0.05 were considered as statistically significant. Statistical analyses were performed using SAS statistical software version 9.2. (SAS Institute Inc., Cary, NC).

Results

Patients were studied at the outpatient clinic of the Division of Nephrology at the University Hospital of Zurich, Switzerland from March 2010 to May 2011. **Table 1** shows the characteristics of 87 CKD patients (mean age 53 years, 52% male) and 20 healthy volunteers serving as controls (mean age 33 years, 60% male). The patients were classified into CKD stages according to the CKD EPI equation:[121] 19% of the participants belonged to CKD stage 1, 22% to stage 2, 13% to stage 3, 23% to stage 4 and 23% to stage 5 (18 (21%) 5D, median dialysis vintage 1.4 years). Causes of nephropathies in CKD patients were: hypertensive nephropathy (n=21, 10% biopsy confirmed), IgA-nephropathy (n=12, 83% biopsy confirmed), diabetic nephropathy (n=6, 33% biopsy confirmed), focal segmental glomerulosclerosis (n=10, 100% biopsy confirmed), Lupus nephritis (n=5, 80% biopsy confirmed), glomerulonephritis (n=13, 46% biopsy confirmed), other kidney diseases (n=16, 81% biopsy-proven), and CKD of unknown etiology (n=5). The frequency of 1,25D and phosphate binder treatment increased with advancing CKD stages. At any CKD stage, approximately half of the patients were supplemented with nutritional vitamin D (**Table 1**). None of the patients were currently or had been treated in the past with bisphosphonates. Healthy volunteers received no medication.

Relationship between serum levels of Klotho, 1,25D, FGF23 and PTH with eGFR

Klotho, 1,25D, FGF23 and PTH levels were plotted versus eGFR and the respective relationships analyzed by the LOWESS function, a statistical technique without assumption on the shape of the relationship.

Figure 1a reveals that serum Klotho and eGFR were associated linearly. Klotho levels continuously declined with progressive degree of severity of renal insufficiency; this fitted a linear model (r^2=0.41, P<0.0001) and it was estimated that the crude mean Klotho level declines by 6.1 pg/ml (95CI 4.7 to 7.5 pg/ml, P<0.0001) as GFR declines by 1 ml/min. We estimated that the mean Klotho change was -5.7 pg/ml (95%CI -4.1 to -7.5 pg/ml, P<0.0001) for each -1 ml/min GFR change adjusted for concomitant medications that possible affect the relationship (r^2=0.42, P<0.0001). In the *post hoc* analysis adjusted for multiple testing by using volunteers as a reference group, Klotho levels were already significantly lower than normal in patients with CKD stage 2 (mean difference -380.6 pg/ml, 95%CI -621.4 to -139.7 pg/ml; P<0.05) and approximately decreased by half from CKD 1 to CKD 5 (mean difference -504.1 pg/ml (95%CI -747.4 to -260.8; P<0.05).

Figure 1b confirms that 1,25D and eGFR are linearly associated and 1,25D levels decline with progressive GFR loss justifying the fitting of a linear model. We estimated that the crude mean 1,25D levels decrease by 0.32 ng/l (95%CI 0.22 to 0.41 ng/l, P<0.0001) as eGFR declines by 1 ml/min. The adjustment for the concomitant medications did only marginally change the estimated 1,25D slope: 0.36 ng/l (0.25 to 0.48; P<0.0001). The *post hoc* analysis revealed that patients at CKD stage 3 to 5 had lower 1,25D levels compared to volunteers.

It is noteworthy that, FGF23 and PTH both showed a plateau at approximately CKD stages 1 to 3 for PTH and 1 to 2 for FGF23 and an exponential increase at higher respective CKD stages in the LOWESS function (**Figures 1c and 1d**),whereas Klotho and 1,25D levels were linearly associated with eGFR **(Figures 1a and 1b).** This fitted a segmented, nonlinear model that consists of two segments connected in a smooth fashion, and we estimated the departure point of the curve from the plateau. The iterative optimization converges after 7 (Klotho vs. eGFR) and 13 iterations (1,25D vs. eGFR), respectively. We estimated that the FGF23 curve departs from the plateau (63.9 RU/ml) at a GFR of 48.6 ml/min (95%CI 41.0 to 56.1 ml/min) whereas the PTH curve departs from the plateau (53.2 ng/ml), at a eGFR of 35.3 ml/min (95%CI 21.7 to 50.0 ml/min). The *post hoc* analysis further confirmed that FGF23 increases earlier in the course of renal insufficiency, compared to PTH: Serum levels of FGF23 in patients at CKD stages 4 and 5 were different from those obtained in healthy volunteers whereas for PTH the serum levels were only different from healthy volunteers at CKD stage 5.

Phosphate, calcium and 25-hydroxy vitamin D

Mean serum phosphate levels increased only modestly in patients at higher CKD stages: 0.96 mmol/l ± 0.17 mmol/l in CKD 1 to 1.69 mmol/l ± 0.48 mmol/l in CKD5 (**Table 2**). Hyperphosphatemia (> 1.1 mmol/l) was present in 46% of the participating subjects, mostly in patients with CKD stages 4 and 5, as expected. The LOWESS function of serum phosphate versus eGFR indicated a plateau at early CKD stages and an exponential increase at advanced CKD stages (**Figure 2a**). The departure point of the curve from the plateau was at an eGFR of 35.1 ml/min (95%CI 21.7 to 49.0 ml/min) estimated by fitting a segmented non linear regression model. The *post hoc* analysis showed that only patients at CKD stage 5 had serum phosphate levels different from those observed in healthy volunteers, similar to what was seen for PTH. The maximal tubular phosphate reabsorption rate (TmP/GFR) remained normal and

unchanged for patients at CKD stage 1 to 3 and then declined at stage 4 (mean difference -0.16 mmol/l, 95%CI -0.31 to -0.01, *post hoc* analysis) **(Table 2).**

The ionized calcium levels remained unchanged across CKD stages 1 to 5 and the LOWESS function remained within the interquartile range (1.19 mmol/l -1.21 mmol/l) of the healthy volunteers (**Figure 2b**). The serum levels of 25-hydroxy vitamin D (25D) also remained unchanged across CKD stages 1 – 5 **(Figure 2C).**

Discussion

The present report is the first illustration of the sequential changes of the serum levels of the 6 key actors which govern mineral metabolism in renal insufficiency, i.e. Klotho, 1,25D, FGF23, PTH, phosphate and calcium: as depicted in **Figure 3**, serum levels of soluble alpha-Klotho and 1,25D decrease in parallel with the progressive decline in glomerular filtration rate, and both do so before a rise in serum levels of FGF23 (at 49 ml/min GFR) followed by a rise in serum levels of PTH (at 35 ml/min GFR) can be detected. Ultimately, i.e. at CKD stage 4 and onwards, serum levels of phosphate start rising, whereas serum levels of ionized calcium remained unchanged across all CKD stages included in this cross-sectional study.

With respect to the chronological sequence FGF23-PTH, these results are in agreement with those recently published by Isakova et al[17] who pointed to the fact that the rise in serum FGF23 does precede the rise in serum PTH in the course of development of renal insufficiency. As to the rise in FGF23 itself in a state of Klotho deficiency, it is thought to be the consequence of phosphorous accumulation as recently demonstrated by preclinical studies which showed absence of said rise in klotho-/- mice fed a low phosphate diet.[22]

The present study illustrates the fact that serum levels of both 1,25D and Klotho decline "hand in hand" with progression of renal insufficiency. Given the current limitations of the respective techniques, it remains speculative to declare at that current stage of knowledge which of both levels declines first, i.e. what is the cause and what is the consequence: on one hand, Tsujikama et al have suggested that Klotho might have an enzymatic ability to modify a receptor or ligand autonomously influencing the activity of 1-alpha hydroxylase; on the other hand, the same authors demonstrated that 1,25D itself modulates the expression of Klotho.[109] Therefore further studies, in particular prospective studies remain to be carrying out to clarify this point.

In summary, we postulate that an initial renal tubular damage leads to down-regulation of expression of both Klotho and 1-alpha-hydroxylase, and that the ensuing cascade comprising the rise in FGF23 and in PTH is the consequence: Indeed, insufficient production of Klotho known to occur at the level of both the parathyroid gland and the distal tubule in condition of renal insufficiency[19, 104] leads to peripheral resistance to FGF23 at both anatomical sites, and FGF23 thus can no longer suppress PTH secretion nor maintain phosphate homeostasis, as already suggested by Kuro-o.[118]

Conclusion

These data elevate the newly assayable serum level of soluble alpha-Klotho to the rank of potentially most sensitive biomarker for early detection of kidney damage; in patients with

various nephropathies it triggers new hopes for effective monitoring of the impact of future therapeutic interventions.

Acknowledgements

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Disclosure

All authors declared no competing interests.

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Figure 1 Scatter plot graphs of A) Klotho, B) 1,25-dihydroxy-vitamin D_3 (1,25D), C) carboxy-terminal fibroblast growth factor 23 (FGF23) and D) intact parathyroid hormone (PTH) versus estimated glomerular filtration rate (eGFR) in chronic kidney disease patients with locally weighted scatter plot smoothing (LOWESS). Each symbol represents one patient.

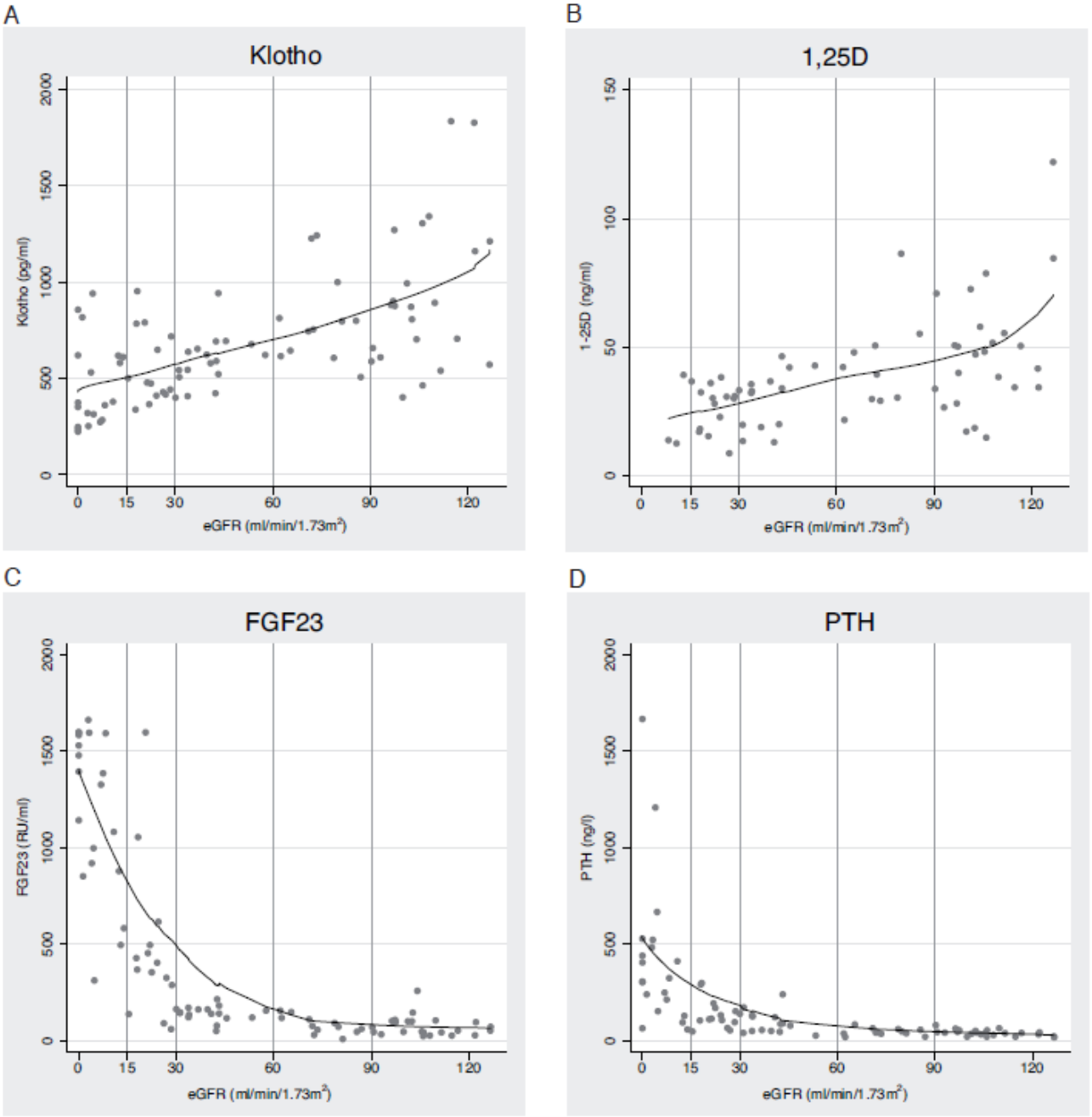
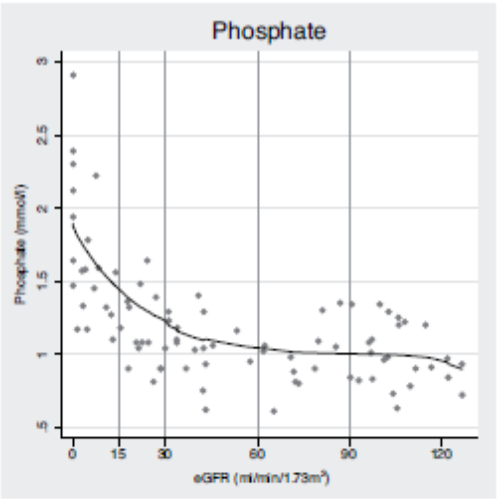
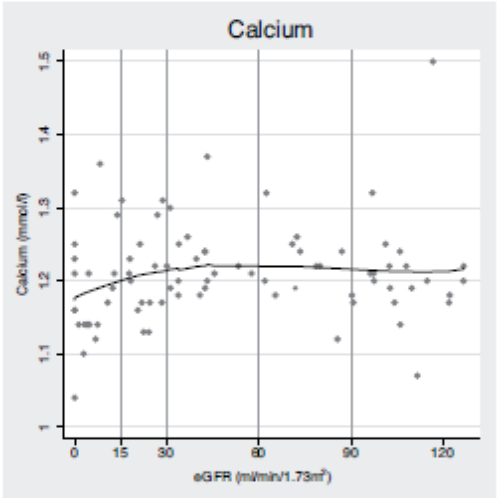


Figure 2 Scatter plot graphs of A) serum phosphate, B) ionized calcium and C) 25-hydroxy-vitamin D (25D), versus estimated glomerular filtration rate (eGFR) in chronic kidney disease patients with locally weighted scatter plot smoothing (LOWESS). Each symbol represents one patient.

A



B



C

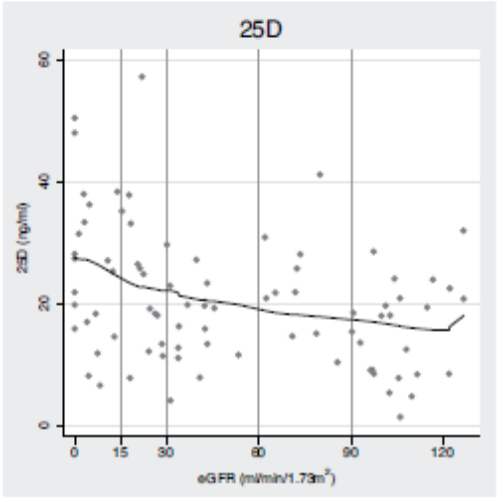


Figure 3 Overlaid LOWESS lines for Klotho, 1,25-dihydroxy-vitamin D_3 (1,25D), carboxy-terminal fibroblast growth factor 23 (FGF23) and intact parathyroid hormone (PTH) versus estimated glomerular filtration rate (eGFR) in chronic kidney disease patients with locally weighted scatter plot smoothing (LOWESS).

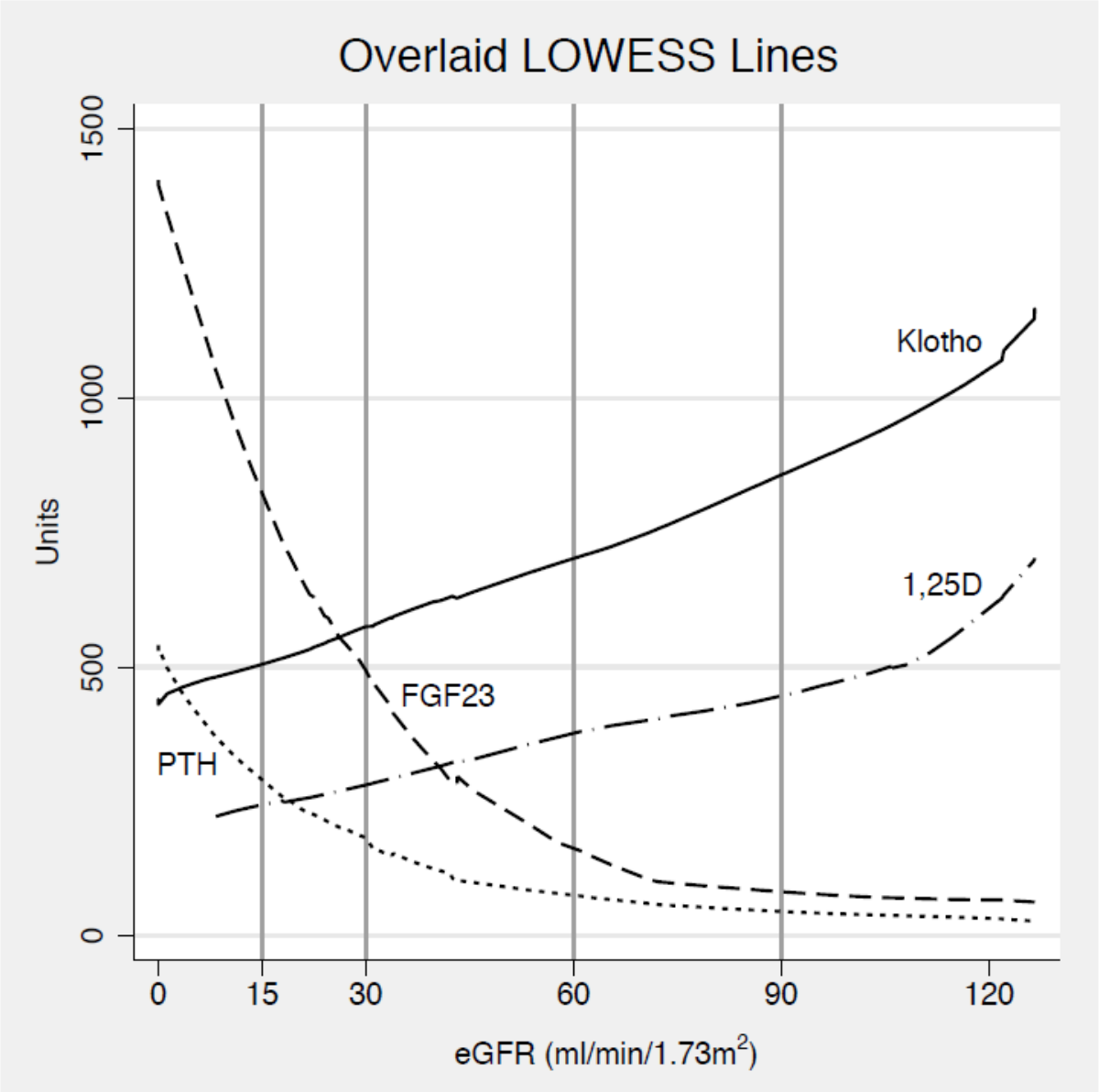


Table 1 Characteristics of healthy volunteers (HV) and of patients with chronic kidney disease (CKD).

	HV N=20	CKD 1 N=17	CKD 2 N=19	CKD 3 N =11	CKD 4 N=20	CKD 5 N=20
Age – years	32 ± 5	41 ± 14	40 ± 12	57 ± 15	64 ± 12	61 ± 17
Sex – no. (%)						
Female	8 (40.0)	7 (41.2)	12 (63.2)	6 (54.6)	9 (45.0)	8 (40.0)
Male	12 (60.0)	10 (58.8)	7 (36.8)	5 (45.4)	11 (55.0)	12 (60.0)
Body mass index - kg per m^2	23 ± 2	28 ± 6	23 ± 5	28 ± 4	28 ± 6	24 ± 6
Creatinine – mg/dl	0.9 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	1.5 ± 0.3	2.5 ± 0.7	6.6 ± 2.8
eGFR - ml/min per 1.73m^2	99.1 ± 11.6	104.1 ± 11.3	81.1 ± 16.5	42.6 ± 6.0	25.3 ± 5.8	5.7 ± 4.9
Blood pressure – mmHg						
Systolic	124 ± 9	139 ± 13	130 ± 13	144 ± 21	145 ± 19	138 ± 26
Diastolic	126 ± 7	83 ± 10	84 ± 8	78 ± 14	79 ± 12	69 ± 21
Medication - no. (%)						
25-hydroxy-vitamin supplement		7 (41.0)	7 (36.8)	9 (81.8)	13 (65.0)	8 (40.0)
1,25-dihydroxy vitamin D_3 treatment		0 (0.0)	1 (5.3)	1 (9.1)	5 (25.0)	4 (20.0)
Calcium-free phosphate binder		0 (0.0)	0 (0.0)	0 (0.0)	2 (10.0)	9 (45.0)
Calcium administration		4 (23.6)	5 (26.3)	1 (9.1)	5 (25.0)	10 (50.0)
Bicarbonate supplement		0 (0.0)	0 (0.0)	0 (0.0)	4 (20.0)	1 (5.0)
Diuretics		1 (5.9)	2 (10.5)	4 (36.4)	14 (70.0)	6 (30.0)
Prednisone		3 (17.7)	6 (31.6)	4 (36.4)	2 (10.0)	3 (15.0)

Abbreviations: eGFR – estimated glomerular filtration rate. Values are means ± standard deviation and numbers (percentage)

Table 2 Parameters of phosphate metabolism in healthy volunteers (HV) and in patients with chronic kidney disease (CKD)

Parameter	HV	CKD 1	CKD 2	CKD 3	CKD 4	CKD 5
	N=20	N=17	N=19	N =11	N=20	N=20
Serum						
Klotho - pg/ml	1200.8 ± 385.0	964.3 ± 398.8	1049.7 ± 1037.5	638.1 ± 128.7	539.7 ± 165.1	460.2 ± 222.8
Q1/Median/Q3	*862.9/1153.1/1371.9*	*703.2/880.1/1159.8*	*623.1/749.1/952.6*	*423.9/622.6/684.9*	*415.1/490.2/640.8*	*282.2/368.3/612.9*
FGF23 - RU/ml	27.5 ± 22.0	89.9 ± 54.3	65.1 ± 41.7	137.7 ± 45.9	377.2 ± 370.3	1200.6 ± 416.4
Q1/Median/Q3	*4.2/27.1/39.2*	*51.1/95.5/101.4*	*38.2/50.8/82.9*	*50.8/140.1/161.3*	*141.6/307.0/434.0*	*910.0/1356.7/1586.4*
PTH - ng/ml	43.3 ± 15.7	43.0 ± 17.0	40.4 ± 16.6	87.4 ± 63.8	129.1 ± 71.2	422.8 ± 392.7
Q1/Median/Q3	*27.2/44.8/58.3*	*32.7/42.3/52.8*	*31.2/39.6/45.9*	*25.9/76.6/88.1*	*87.2/119.4/156.7*	*196.9/314.2/492.4*
25-hydroxy-vitamin D - µg/l	25.4 ± 12.0	15.1 ± 8.9	20.4 ± 8.2	17.6 ± 6.0	21.9 ± 12.5	25.9 ± 12.4
Q1/Median/Q3	*19.6/22.3/29.2*	*8.5/15.4/20.8*	*14.7/20.9/24.0*	*7.9/19.3/19.8*	*12.7/18.8/27.3*	*16.7/26.2/34.1*
1,25-dihydroxy-vitamin D_3 - ng/l	51.2 ± 10.0	48.5 ± 25.9	45.7 ± 19.6	31.7 ± 12.7	27.12 ± 8.7	21.8 ± 15.0
Q1/Median/Q3	*45.0/49.6/57.2*	*34.3/41.6/55.4*	*29.8/48.0/51.7*	*13.0/35.4/42.3*	*19.3/30.4/33.0*	*13.2/13.8/26.5*
Phosphate - mmol/l	1.01 ± 0.12	0.96 ± 0.17	1.02 ± 0.24	1.01 ± 0.22	1.15 ± 0.21	1.69 ± 0.48
Q1/Median/Q3	*0.92/0.98/1.10*	*0.84/0.96/1.08*	*0.85/1.02/1.24*	*0.62/1.03/1.11*	*1.04/1.09/1.30*	*1.33/1.58/1.99*
Ionized Calcium - mmol/l	1.20 ± 0.02	1.20 ± 0.05	1.24 ± 0.09	1.23 ± 0.05	1.22 ± 0.06	1.19 ± 0.08
Q1/Median/Q3	*1.19/1.20/1.21*	*1.18/1.20/1.21*	*1.20/1.22/1.25*	*1.18/1.22/1.24*	*1.17/1.21/1.25*	*1.14/1.17/1.22*
Spot Urine						
Phosphate - mmol/l	22.9 ± 13.6	12.8 ± 5.8	16.5 ± 12.9	9.7 ± 4.6	12.5 ± 5.4	-
Q1/Median/Q3	*13.9/21.3/30.0*	*8.1/13.1/16.1*	*4.1/15.3/21.2*	*5.4/8.7/11.2*	*9.4/11.3/15.9*	
TmP/GFR - mmol/l/GFR	0.88 ± 0.12	0.85 ± 0.16	0.89 ± 0.23	0.86 ± 0.25	0.72 ± 0.16	-
Q1/Median/Q3	*0.79/0.86/0.99*	*0.74/0.81/0.97*	*0.69/0.85/1.13*	*0.42/0.91/1.03*	*0.60/0.78/0.85*	

Abbreviations: Q1 - 0.25 quartile; Q3 - 0.75 quartile, 1,25-dihydroxy-vitamin D_3 (1,25D), FGF23 - carboxy-terminal fibroblast growth factor 23, PTH - intact parathyroid hormone, TmP/GFR - Tubular maximum phosphate reabsorption per ml of glomerular filtrate.

9 Conclusion

The optimal control of mineral ion metabolism and hormonal factors, as PTH secretion and Vitamin D regulation, are crucial for the therapy and treatment of chronic kidney disease patients. In the past years a global initiative has tried to provide guidelines regarding renal disease patients considering a variety of different demographics. This global initiative has been founded as the National Kidney Foundation Disease Outcomes Quality Initiative (NKF KDOQI) and has ever since provided evidence-based clinical practice guidelines for all stages of chronic kidney disease (CKD) and related complications since 1997. Acknowledged as a foundation to update based on clinical trial outcome and knowledge generated via scientific efforts, these guidelines have been improving the diagnosis and treatment of kidney disease throughout the world. The KDOQI Guidelines have changed the practices of numerous specialties and disciplines and improved the lives of thousands of kidney patients.[122]

Nevertheless the renal community has come to an agreement that current data obtained via clinical trials lack to provide hard fact based evidence. Most of the trial data available are post hoc observational data collected from various registries over the years.[123] The field of renal disease research is lacking well designed prospective interventional trials to provide the answers needed regarding questions to influence treatment outcome and therapy choices, per example what the optimal levels are for PTH, Ca and Pi to avoid either adynamic bone disease or vascular calcification. It is very limiting to use observational trial data to draw causality and effect. The field of renal disease research has come to an agreement that basic

science findings have gained the respect to be further investigated in a clinical setting, opening the field to a couple of new screening parameters, as an example phosphate homeostasis. These factors have been mainly identified as phosphaturic hormones FGF23 and [103]available for a longer period of time, hence most of the research has been conducted in house and data collected has been only shared on closed communities. Since early 2000 commercially available antibodies against these specific hormones have provided a valid possibility to measure these parameters, qualifying and quantifying them for a clinical setting. Although they have not reached complete validation as screening parameters in daily clinical practice; their importance based on basic science data has been fully acknowledged. FGF23 has been the most important factor to draw the essential line called bone kidney axis, leading to confirmation that renal insufficiency has the consequence of negatively impacting multiple organs very early in disease. Furthermore the importance of FGF23 has gained a wide field of impact, associating it with higher levels of mortality, vascular calcification, and mineral bone disease, just to name a few. The knowledge gained, associating FGF23 with specific pathophysiological phenotypes has been broadened over the past 10 years, however, we have not yet established what the exact levels of harm are versus how much FGF23 is actually needed to maintain and control phosphate in the system. Since specifically in the past two years we have learned a great deal regarding the specific pathways and activation of FGF23 receptor and the way they regulate in order to avoid excessive levels of phosphate and vitamin D and their resulting toxic effects.[124] This knowledge has to be taken further investigating the

levels of FGF23 in specific clinical patient cohorts. Research efforts focusing on using FGF23 as a therapy target will provide further clarification.

In the early nineties, the discovery of Klotho has impacted the view on the renal pathophysiology in an essential way. What started as a discovery of an anti aging molecule has shortly after been associated with renal insufficiency. Since the Klotho knockout mice present a highly similar phenotype as chronic kidney disease patients. The detection of Klotho in various tissues has provided insight in a wide range of functionalities of Klotho as a paracrine Proteohormone. The definition as a Proteohormone, is based on the fact that Klotho as a single transmembrane domain protein, with a c-terminal extracellular domain that when cleaved acts as a paracrine hormone. The Klotho knockout phenotype in mice shows a high similarity to the FGF23 knockout phenotype which has led to the association that additional to its anti aging features; Klotho has the ability to act as a co factor to FGF23. The cleaved c-terminal domain of Klotho, which acted as a paracrine hormone showed to have the tubular epithelial cell as a target. The tubular epithelia cells are at the same time the location where FGF23 receptor activation occurs, leading to the signaling pathway initialized via proper binging to the receptor. Early in the discovery of the 23rd member of the FGF family it has been established that FGF23 needs for accurate binding a heparin/sulfate cofactor; solely it can only peripherally bind to the receptor, initializing only weak and improper activation. As Klotho has been determined to be expressed in different tissues, it has been of great interest to evaluate its function and signaling pathway as a circulating hormone (soluble α-Klotho). Klotho has the molecular features to form with FGF23 a tertiary complex that as a construct

has the conformational ability to bind with high affinity to the FGFR1c receptor and initialize proper signaling. The signaling pathways impact on the phosphate transporters in the proximal tubules, including renal phosphate wasting, due to suppression of phosphate reabsorption. Specifically the action on the Na/Pi co-transporters has been studied in relation to the FGF23/Klotho complex. The results obtained have identified that Klotho is essential for FGF23 to fulfill its endocrine biological activity. On the other hand, Klotho has the ability to act as an individual factor with no co-dependence on another molecule or hormone. Specifically its ability to retain calcium when overexpressed via activation of the TRPV5b channel and its influence on the activation of Vitamin D. Overall FGF23 and Klotho have been recognized as potential factor to elucidate the pathophysiology in early chronic kidney disease.

There are in general a smaller number of patients with CKD stages 1-2, than stages 3 – 5, mostly because the symptoms of renal failure are established over a longer period of time in silence. This small number has the implication that evidence based research is lacking hard facts to fully establish the picture how exactly renal failure starts in dependence of hormonal and mineral ion factors.

Kuro et al. have hypothesized that Klotho has the potential to be the earliest biomarker to implicate potential chronic renal failure, since it has been shown that it is the first factor to become imbalanced when renal disease starts.

This hypothesis plays an important role since the stages of CKD 1 – 2 have not been elucidated enough due to late capturing of the disease progress in daily clinical practice. It is yet to be clarified what the implication would be regarding certain levels of Klotho in relation to disease outcome, forecasting 10 years.

10 Outlook

This doctoral thesis focused exclusively on the expression and implications of phosphaturic hormones and parameters involved in phosphate homeostasis in a human setting. The findings and conclusions of this doctoral thesis are solely based on a clinical research effort.

For future purposes to elucidate the role and regulation of both phosphaturic hormones, Klotho and FGF23, in the development and outcomes of CKD and ADPKD, and in order to establish them in a potential therapeutic setting, it is of essence to further study their molecular mechanisms and regulatory pathways in a basic research setting.

Focusing on ADPKD there are currently two well established rodent models that can be used to further study FGF23 expression patters and regulatory functions; the HanSPRD rat and the PKD mouse model. Since we already established that FGF23 expression levels reach a significantly high level in our ADPKD cohort independently of their age or glomerular filtration rate, research on the HanSPRD rat could clarify the origin of expression of FGF23 in ADPKD. Current opinion reached an agreement that FGF23 is sequestered by the osteocytes, yet it would be of great interest when in the setting of PKD the bone reacts with increasing the expression levels and under which circumstances this can be manipulated. Observation should involve as well implication to bone mineral density and bone development and later turn over regarding FGF23 expression. One could study the implications of FGF23 while suppressing FGF23 on genetic levels via knock out or blunting the FGF23 function with an FGF23 neutralizing antibody. Since we could agree that FGF23 activity could not be studied solely, yet in relation to Klotho acting as his co-factor for increasing receptor affinity and leading to full activation of the receptor. It is of essence to study Klotho expression both in tissue as well

as soluble α-Klotho levels acting as an endocrine hormone. Since our findings pointed to the possible hypothesis that the FGF23 resistance established in our ADPKD patients might be due to the lack of Klotho circulating in serum.

It is important that these studies would need to be conducted in both models: the HanSPRD rat model, and the PKD mouse. Simply due to the fact that the cyst development in the PKD mouse model resembles more closely the cyst development in a human setting, meaning that the cyst development is initiated on the level of the distal convoluted tubule, whereas cyst development in the HanSPRD rat originates primarily from the proximal tubules. We postulated in regards to our findings, that the lower levels of Klotho in our Human ADPKD patients might be secondary to the patients' cyst development and the destruction of the endothelial cell architecture on the levels of the distal convoluted tubule; bearing in mind that the transmembrane Klotho expression is located on the level of the distal convoluted tubule.

Early in the course of scientific research on the phosphaturic hormones the question came up if FGF23 is a friend or a foe? And Klotho gained great respect to be implied with anti-aging. As both act as either paracrine or endocrine hormones it is a fact that they are influencers and complete suppression of both leads to pathophysiological states as described earlier. In the case of ADPKD and its specific manifestations in regards to high levels of FGF23 and lower levels of Klotho in early stages of the disease it would be very interesting to study on one hand the exposure of high levels of FGF23 long term and the implication on the bone. One could postulate that under constant FGF23 accumulation the bone lowers the production. This down regulation might go along with the bone starting to lower its turnover. On the other

hand, suppressing of Klotho completely in ADPKD might lead to complete resistance of FGF23 and accumulation of phosphate leading to possible vascular calcification and hypertension. More possibly since Klotho suppression was associated with calcium accumulation in serum. Overall long term exposure to either excessive exposure of complete suppression in regards to both FGF23 and Klotho would provide further clarification regarding calcium/phosphate homeostasis and kidney bone axis.

We have implied that further knowledge regarding the role and regulation of these phosphaturic hormones might as well lead to contribution to novel therapeutic approaches and here it needs to be stated that Klotho as a individual player and co-factor to FGF23 might be the more promising therapeutic tool, meaning to be supplemented to avoid FGF23 accumulation and maintain the phosphate homeostatic function in association with FGF23. To conclude, before a therapeutic approach might be considered, the position of FGF23 and Klotho as sensitive markers regarding maintenance of mineral homoeostasis in various chronic kidney disease states needs to be further established.

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12 Appendix

12.1 Co-Authorships

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Safety and tolerability of sirolimus treatment in patients with autosomal dominant polycystic kidney disease

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Abstract

Background. We initiated a randomized controlled clinical trial to assess the effect of sirolimus on disease progression in patients affected by autosomal dominant polycystic kidney disease (ADPKD). Here we report the preliminary safety results of the first 6 months of treatment.

Method. A total of 25 patients were randomized to sirolimus 2 mg/day and 25 patients to no treatment except standard care. Treatment adherence was monitored electronically. At baseline and at Month 6, laboratory parameters were analysed and the urinary protein profile in 24-h urine collections was determined.

Results. Both treatment groups were well balanced for age, sex and renal function. In 94.1 ± 11.4% of the study days, patients in the sirolimus group were exposed to the drug when assuming a therapeutic efficacy duration of 30 h. At Month 6, the mean sirolimus dose and trough level were 1.28 ± 0.71 mg/day and 3.8 ± 1.9 μg/l, respectively. Glomerular (albumin, transferrin, IgG) and tubular (retinol-binding protein, α_1-microglobulin) protein excretion remained unchanged. Glomerular filtration rate also did not change significantly. Haematological parameters were similar in both groups, except for a mild reduction of the mean corpuscular volume of erythrocytes in patients receiving sirolimus. Lipid levels were similar in both groups. Adverse events were transient and mild, and no grade 3 or 4 events occurred. The incidence of infections was similar in the sirolimus group (80%) and the standard group (88%). The most common gastrointestinal adverse events were mucositis (72% in the sirolimus group versus 16% in the standard group, $P = 0.0001$) and diarrhoea (36% in the sirolimus versus 20% in the standard group, $P = 0.345$).

Conclusion. Treatment of ADPKD patients with sirolimus with a dose of 1–2 mg/day is safe and does not cause proteinuria or impairment of GFR. Treatment adherence was excellent. (ClinicalTrials.gov number, NCT00346918.)

Keywords: autosomal dominant polycystic kidney disease (ADPKD); safety; sirolimus; treatment adherence; urinary protein profile

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) represents the most frequent potentially lethal monogenic hereditary disease of mankind [1]. The estimated number of cases in Europe and in the United States amounts to 700 000 and 300 000, respectively. The relentless development and growth of innumerable cysts lead to progressive destruction of the normal renal parenchyma and massive enlargement of the kidneys. Subsequently, the glomerular filtration rate decreases in an accelerated mode, and end-stage renal disease with the need for dialysis and/or transplantation ensues. Data from the consortium for radiologic imaging studies of polycystic kidney disease (CRISP) have shown that the rate of kidney volume growth is an excellent predictor of renal functional decline [2]. Therefore, kidney volume growth can be used as a surrogate marker of disease progression [3]. Despite decades of intense basic and clinical research, effective treatment that alters the course of ADPKD has not been established.

Sirolimus, also termed rapamycin, is an immunosuppressant that binds to FK-binding protein-12 (FKBP-12) and inhibits the activation of mTOR, a key regulatory kinase of growth and proliferation [4]. We and others have previously shown that the mTOR inhibitors sirolimus and everolimus effectively reduce cyst growth and loss of renal function in an experimental animal model for polycystic kidney disease (PKD) [5–7]. Additional studies have shown that sirolimus is also effective in various mouse models of PKD, including dominant and recessive forms [8]. Of interest, analyses of ADPKD patients that received a renal allograft revealed that cystic kidney and liver volumes regressed under immunosuppression with sirolimus [8,9].

Based on these encouraging studies, we have initiated a randomized controlled clinical trial as a proof-of-concept study to examine the effect of sirolimus on cyst volume growth in young patients with documented ADPKD and normal GFR [10]. Here we report the preliminary safety and tolerability results of this clinical trial, with particular emphasis on the effect of sirolimus on proteinuria and GFR.

Methods

Patient population and study design

The SUISSE ADPKD study represents an ongoing single-centre, prospective, open-label, randomized controlled clinical trial to assess the efficacy of sirolimus (Rapamune®, Wyeth AG, Zug, Switzerland) to decrease renal volume enlargement in patients with ADPKD. The study involves 100 ADPKD patients aged 18–40 years with an estimated creatinine clearance >70 ml/min. Prior to randomization, polycystic kidneys are visualized by MRI without contrast media within an interval of 6 months, and kidney and cyst volumes are measured by volumetry. The volumetry method has been previously validated and showed an excellent reliability [3]. Patients with documented total kidney volume (TKV) enlargement of >2% are randomized at a one-to-one ratio of sirolimus 2 mg/day or standard care for 18 months. At the discretion of the treating physician, the starting dose was reduced to 1 mg in case of anticipated sirolimus-associated toxicity. An independent biostatistics unit generated the randomization list, using a permuted block design with a random block size of 4 or 6 to guarantee a balanced allocation. Patients in the standard arm had visits at Months 3 and 6, and patients in the sirolimus arm had four extra visits at Weeks 2 and 4 and Months 1 and 2 after randomization to allow for blood level monitoring and dose adjustments. The sirolimus dose was reduced or withheld when the trough level exceeded 10 μg/l, or when elevated liver enzymes (>2-fold above normal values), thrombopenia (<100 000/mm^3) and leukopenia (<3000/mm^3) occurred. Standard care included blood pressure control and symptomatic treatment of flank pain, cyst bleedings and cyst infections.

The study was approved by the institutional review board, conducted according to Good Clinical Practice guidelines and the Declaration of Helsinki and controlled by external monitoring. All participants gave written informed consent. Details of the study design have been reported elsewhere [10]. Briefly, from a local prospective ADPKD cohort, we selected 100 patients with documented TKV progression for inclusion (i.e. randomization) in the trial. Approximately 80% of patients considered for randomization had ≥2% TKV progression within an interval of 6 months (data not shown). According to a predefined interim analysis plan, the first 50 patients that had completed 6 months of sirolimus treatment ($n = 25$) or standard care ($n = 25$) were subjected to a safety and tolerability analysis. The entire study is powered to enrol a total of 100 patients and to continue until there is a mean 18-month follow-up. Here we report on the first 50 randomized patients, focusing particularly on the safety and tolerability of sirolimus treatment.

Study procedures

A detailed medical history was obtained from all patients, including ADPKD-related symptoms, previous hospitalizations and medication. Blood pressures were measured twice 5 min apart in each arm in the sitting position after a rest of 5 min, using an oscillometric blood pressure monitor (Boso-Medicus, Jungingen, Germany) at each visit. The lower of the two consecutive measurements in the arm with the higher blood pressure was used for analysis. Arterial hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg on two or more study visits, or treatment with an antihypertensive drug.

Adherence to the prescribed study drug was assessed during the entire treatment period by an electronic system that monitors the date and time of the medication bottle opening (MEMS®, Aardex Ltd, Zug, Switzerland). This electronic system reliably assesses medication adherence as a period with a lack of medication bottle opening, which was considered to represent an episode of non-adherence [11,12]. The percentage of time when the patient was exposed to the drug action was calculated based on two different estimates of duration of therapeutic efficacy of sirolimus, namely 24 h plus 6 h (6-h forgetfulness period) and 24 h plus 24 h (24-h forgetfulness period).

Laboratory analyses

Blood was obtained for the determination of creatinine (IDMS-traceable modified Jaffé method), lipids, liver enzymes and haematologic parameters including erythrocyte indices. Trough levels of sirolimus were determined by liquid chromatography—mass spectrometry in samples of whole blood.

Twenty-four-hour urine samples were collected at baseline and at Month 6. Aliquots of these urine samples were centrifuged at 1500 *g* for 5 min, and the supernatants were stored at −80°C prior to analysis. Albumin, transferrin and immunoglobulin G (IgG) were analysed as urinary markers of glomerular damage, and retinol-binding protein (RBP) and α_1-microglobulin as urinary markers of tubular damage. Measurement of urinary total protein (benzethonium chloride method, Roche Ltd, Basel, Switzerland) and serum creatinine (enzymatic method, Wako Pure Chemical Industries Ltd, Osaka, Japan) were performed on a Hitachi 917 analyser, while urinary marker proteins were measured on a Beckman Coulter nephelometry system (Beckman Coulter, Brea, USA) using antibodies against albumin, transferrin, IgG and α_1-microglobulin (Beckman Coulter) and RBP (Dako, Glostrup, Denmark). The interassay coefficient of variation was <5% for these assays. The lower limit of quantification of the assays was 0.04 g/l for total protein, 2.0 mg/l for albumin, 0.61 mg/l for transferrin, 3.0 mg/l for IgG, 0.328 mg/l for RBP and 4.0 mg/l for α_1-microglobulin [13]. The concentration values in urine samples below the lower limit of quantification were set to zero for all calculations.

Safety assessment

Safety was determined on the basis of the occurrence of adverse events, findings on physical examination, and laboratory evaluations. Adverse events affecting ≥5% in either group were described and graded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), version 3.0 [14]. A patient with multiple occurrences of an adverse event was counted once in the corresponding category and a patient with multiple adverse events within a primary system organ class was also counted once for that class. Primary system organ classes and preferred terms were sorted by the frequency of adverse events in the total group. Safety and tolerability analyses included all randomized patients who received at least one dose of sirolimus and underwent at least one safety assessment.

Statistics

The results were expressed as means ± standard deviation or number of patients (percent). For comparisons between groups, means of continuous data were compared using Student's *t*-test or the Wilcoxon rank-sum test as appropriate and categorical data using Fisher's exact test. All *P*-values were two sided for the comparison between the groups or between baseline and follow-up values, and those <0.05 were considered statistically significant.

Results

Characteristics of the patients

Here we report on 50 ADPKD patients who were enrolled from March 2006 to March 2007 and have completed the first 6 months of the treatment. A total of 25 patients were randomly assigned to receive sirolimus and 25 patients to receive no treatment except standard care. Table 1 shows that both treatment groups were well balanced at baseline for age, sex, body mass index (BMI) and blood pressure. Approximately 70% of patients in each group had hypertension, mostly treated with angiotensin-converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARB). None of the patients were lost to follow-up.

Table 1. Characteristics of the patients at baseline[a]

Characteristic	Sirolimus $N = 25$	Standard $N = 25$	P-value
Age (years)	29 ± 6	29 ± 6	0.964
Gender, No. of male patients (%)	14 (56)	20 (80)	0.069
BMI (kg/m^2)	24 ± 4	25 ± 4	0.284
Systolic blood pressure (mmHg)	131 ± 16	131 ± 15	0.985
Diastolic blood pressure (mmHg)	87 ± 11	83 ± 10	0.122
Hypertension, No. (%)	17 (68)	17 (68)	1.000
Antihypertensive treatment, No. (%)			
All	10 (40)	12 (48)	0.776
ACEi and/or ARB therapy	8 (32)	11 (44)	0.561
Diuretics	2 (8)	5 (20)	0.417
Others	3 (12)	3 (12)	1.000
Symptoms and complications of ADPKD, No. (%)			
History of flank pain	6 (24)	15 (60)	0.021
History of macrohaematuria	6 (24)	6 (24)	1.000
History of cyst infections	1 (4)	3 (12)	0.609
History of intracranial bleeding	0 (0)	1 (4)	1.000
≥2 complications of ADPKD	3 (12)	4 (16)	1.000
Family history of intracranial bleeding	3 (12)	4 (16)	1.000

[a]Table shows either the mean ± standard deviation or the number of patients (percent). BMI denotes body mass index, ACEi angiotensin-converting enzyme inhibitor and ARB angiotensin receptor blocker.

Study drug adherence

The patients in the sirolimus group were exposed to the drug in 94.1 ± 11.4% of the study days if the duration of therapeutic efficacy was assumed to be 30 h, or in 96.6 ± 9.7% of the days assuming a therapeutic drug action of 48 h. The percentage of days with correct dosing was 93.0 ± 12.1%. A total of 17 patients received the correct sirolimus dose in >95% of the study days assuming 30 h of sirolimus therapeutic duration. The number of patients with correct dosing at >95% of the study days amounted to 22 patients when the therapeutic efficacy duration of sirolimus was set to 48 h.

Sirolimus dose and whole blood trough levels

Figure 1 shows the sirolimus dose and sirolimus whole blood trough levels. The starting dose was reduced to 1 mg/day in nine patients, mostly females (eight females, one male), due to anticipated more severe side effects in young female ADPKD patients. After adjustment of the sirolimus dose in the first 2 months, the sirolimus dose, dose per body weight and whole blood trough levels remained constant and after 6 months amounted to 1.28 ± 0.71 mg/day, 0.018 ± 0.01 mg/kg/day and 3.8 ± 1.9 µg/l, respectively.

Effect of sirolimus on renal function and urinary protein excretion

Renal function assessed by measured and estimated creatinine clearance as well as serum creatinine was similar in the sirolimus and standard groups at baseline and at Month 6 (Table 2). Thus, sirolimus did not adversely affect GFR.

Since it is well known that sirolimus can cause proteinuria in patients with preexisting renal disease, we examined in detail the urinary protein profile in 24 h-urine collections (Table 2). The median urinary excretion of total protein was low, and similar in both groups at baseline (78.0 mg/day in the sirolimus group versus 65.0 mg/day in the standard group, $P = 0.077$) and at Month 6 (120 mg/day in the sirolimus group versus 86.3 mg/day in the standard group, $P = 0.087$). The median urinary excretion of albumin was low in both groups at baseline (13.7 mg/day in the sirolimus group versus 9.0 mg/day in the standard group, $P = 0.450$) and remained low in response to sirolimus treatment at Month 6 (36.0 mg/day in the sirolimus group versus 18.9 mg/day in the standard group, $P = 0.349$, Figure 2). The number of patients developing microalbuminuria (as defined by urinary albumin excretion >30 mg/24 h) during the 6-month interval was similar in each group (+3 patients in the sirolimus group versus +2 patients in the standard group), and none of the patients had macroalbuminuria (defined by urinary albumin excretion >300 mg/24 h) at any time point. Furthermore, the urinary excretion of transferrin did not change significantly during the 6-month interval. The changes in the urinary protein excretion that occurred within the 6-month interval were similar in each group (sirolimus versus standard mean changes of albumin +8.4 mg/24 h, $P = 0.543$; transferrin +0.18 mg/mmol, $P = 0.791$; and total protein −1.8 mg/24 h, $P = 0.938$). IgG was detectable in four and two urine samples of the control group and in two and seven urine samples of the sirolimus group at baseline and at Month 6, respectively.

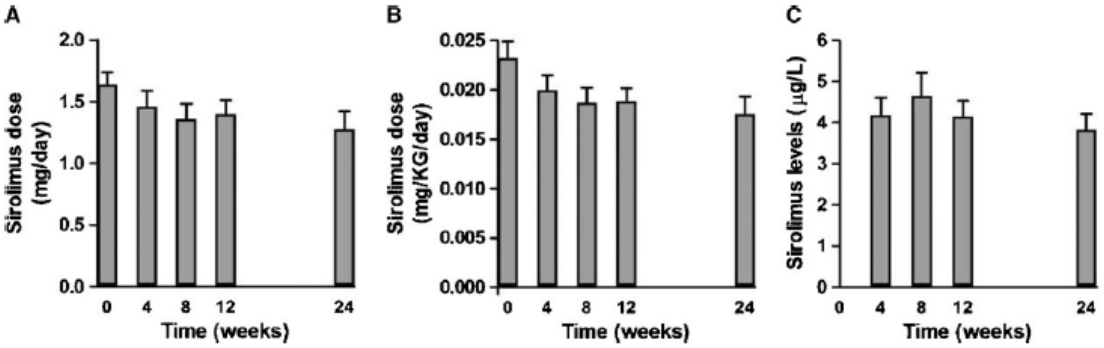

Fig. 1. Mean sirolimus dose (**A**), sirolimus dose per body weight (**B**) and sirolimus whole blood trough levels (**C**) at baseline and at follow-up visits.

Table 2. Changes in renal function and urinary protein excretion from baseline to Month 6[a]

Characteristic	Units	Normal range	Baseline			Month 6		
			Sirolimus $N = 25$	Standard $N = 25$	P-value	Sirolimus $N = 25$	Standard $N = 25$	P-value
mCrCl (24-h urine collection)	ml/min	90–120	91.7 ± 42.7	92.7 ± 46.7	0.937	110.1 ± 31.8	110.0 ± 30.7	0.996
eCrCl (Cockcroft–Gault formula)	ml/min	90–120	105.1 ± 25.4	114.1 ± 23.0	0.195	109.2 ± 24.1	113.9 ± 21.7	0.474
Serum creatinine	µmol/l	62–106 men 44–80 women	90.5 ± 18.3	95.4 ± 18.1	0.345	87.3 ± 15.7	96.2 ± 17.5	0.065
Urinary excretion (24-h urine collection)								
Albumin, median (IQR)	mg/24 h	<20	13.7 (7.4–25.2)	9.0 (4.0–27.2)	0.450	36.0 (12.0–65.0)	18.9 (9.2–46.7)	0.349
Transferrin, median (IQR)	mg/24 h	<1.7	0.87 (0.00–3.00)	0.00 (0.00–1.84)	0.280	2.04 (1.26–3.86)	1.48 (0.00–2.63)	0.273
Total protein, median (IQR)	mg/24 h	<100	78.0 (60.0–123.0)	65.0 (42.0–92.0)	0.077	120.0 (84.0–175.0)	86.3 (73.9–114.0)	0.087
IgG levels above LLQ, No. (%)			2 (8)	4 (16)		7 (36)	2 (8)	0.138
RBP levels above LLQ, No. (%)			0 (0)	0 (0)		0 (0)	1 (4)	1.000
A1M levels above LLQ, No. (%)			2 (8)	3 (12)		4 (16)	6 (24)	0.715

[a]Table shows either the mean ± standard deviation or number of patients (percent), with the exception of urinary albumin, transferrin and total protein excretion values, which are given as medians with interquartile range because of skewed data distribution.

mCrCl, measured creatinine clearance; eCrCl, estimated creatinine clearance; CG, Cockcroft–Gault; IQR, interquartile range; IgG, immunoglobulin G; LLQ, lower limit of quantification; RBP, retinol-binding protein; A1M, α_1, microglobulin.

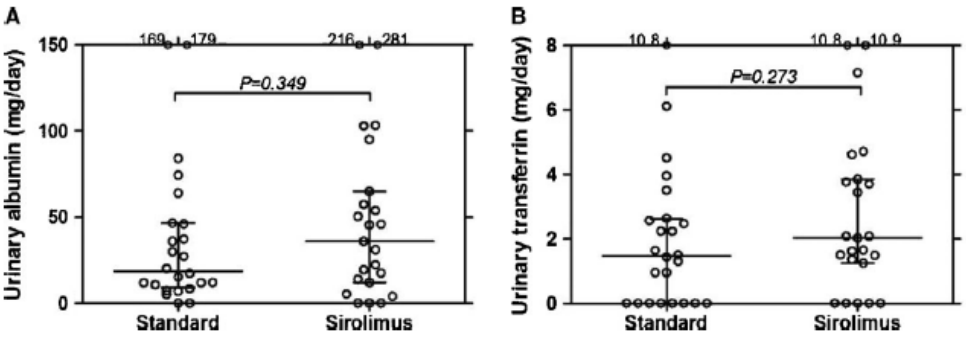

Fig. 2. The amount of albumin (**A**) and transferrin (**B**) in 24-h urine collections at Month 6. Lines represent the median and interquartile range.

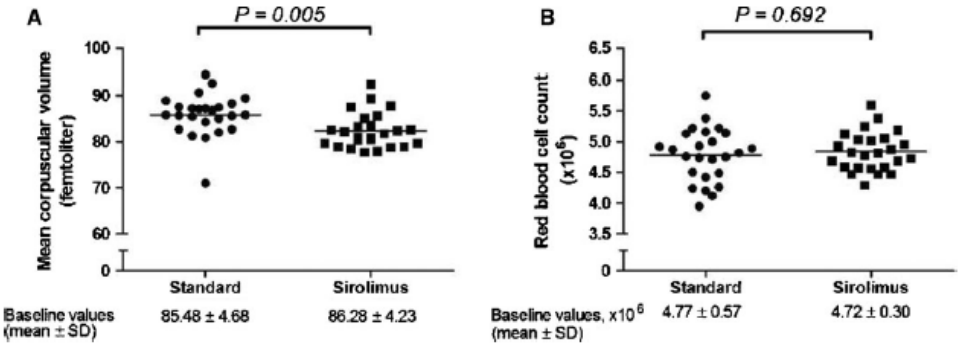

Fig. 3. Mean corpuscular volume of erythrocytes (**A**) and red blood cell count (**B**) at Month 6. The lines represents the mean. Baseline mean values ± standard deviations are shown below the *x*-axis.

Regarding the tubular proteins, RBP was detectable at low concentration only in one urine sample at Month 6 and was below the lower limit of quantification in all other samples. Likewise, α_1-microglobulin was only detectable in three and six urine samples of the standard group and in two and four urine samples of the sirolimus group at baseline and at Month 6, respectively. In urine samples of 10 patients of the standard group and of 9 patients of the sirolimus group, RBP or IgG or α_1-microglobulin was detectable at any time point of the study.

Taken together, the urinary excretion of markers for glomerular and tubular damage remained unchanged during the 6-month interval and was not adversely affected by sirolimus treatment.

Effect of sirolimus on clinical and laboratory parameters

Table 3 shows that the BMI and blood pressure were similar in the sirolimus and in the standard group at Month 6. We also analysed the influence of sirolimus on the antihypertensive treatment. At baseline, 40% patients in the sirolimus group and 48% patients in the standard group received an antihypertensive medication, mostly ACEi or ARB. The number of patients that started a new ACEi or ARB treatment during the 6-month treatment phase (30% of the sirolimus group versus 10% of the standard group) was also similar.

Sirolimus can cause haematological alterations in renal transplant recipients. The mean haemoglobin, mean corpuscular haemoglobin concentration (MCHC), and leukocyte and platelet counts did not differ significantly in the two groups of ADPKD patients (Table 3). The red blood cell count was unchanged, whereas, the mean corpuscular volume (MCV) and the mean corpuscular haemoglobin (MCH) were significantly lower in patients receiving sirolimus compared to patients in the standard group (Figure 3). Also the percentage of microcytic erythrocytes was higher in patients on sirolimus (1.3 ± 2.3% in the standard group versus 2.2 ± 1.3% in the sirolimus group, $P = 0.002$).

The mean aspartate aminotransferase (AST) value was significantly higher in patients receiving sirolimus at Month 6. However, all values of individual patients remained below the 2-fold upper limit of the normal range in either group. The mean alanine aminotransferase and gamma-glutamyltransferase values were similar in both groups.

Sirolimus is known to cause hyperlipidaemia. The mean cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglyceride levels did not differ significantly among the groups (Figure 4). The ratio of LDL to HDL cholesterol was also similar among the groups. Although sirolimus treatment was associated with a tendency to higher values of triglyceride and cholesterol, the range of values and numbers of patients above the pre-defined cut-offs were similar in both groups at Month 6. One patient started a statin treatment in the sirolimus group and none in the standard group.

Table 3. Relevant clinical and laboratory data at Month 6[a].

Parameter	Sirolimus ($N = 25$)	Standard ($N = 25$)	P-value
BMI (kg/m^2)			
Mean ± SD	24 ± 4	25 ± 3	0.314
Range	18–30	17–35	
Systolic blood pressure (mmHg)			
Mean ± SD	126 ± 15	134 ± 12	0.054
Range	96–156	103–160	
Diastolic blood pressure (mmHg)			
Mean ± SD	83 ± 8	85 ± 9	0.505
Range	60–101	70–102	0.702
Antihypertensive treatment, No. (%)			
All	15 (60)	15 (60)	1.000
ACEi and/or ARB therapy	14 (56)	14 (56)	1.000
Diuretics	3 (12)	5 (20)	
Others	3 (12)	3 (12)	1.000
Haemoglobin (g/l)			
Mean ± SD	139.4 ± 8.49	144.2 ± 11.19	0.097
Range	118–154	128–166	
MCV (fl)			
Mean ± SD	82.40 ± 3.81	84.12 ± 4.47	**0.005**
Range	77.80–92.50	71.00–94.40	
MCH (pg/cell)			
Mean ± SD	28.88 ± 0.64	30.25 ± 1.91	**0.009**
Range	26.50–32.60	23.60–32.50	
MCHC (g/dl)			
Mean ± SD	35.04 ± 2.15	35.24 ± 1.34	0.465
Range	32.10–36.90	33.20–37.20	
Leukocyte count ($\times 10^3$/mm^3)			
Mean ± SD	6.03 ± 2.06	5.81 ± 1.64	0.673
Range	3.0–12.2	3.9–12.2	
Platelet count ($\times 10^3$/mm^3)			
Mean ± SD	253.7 ± 63.11	253.3 ± 53.70	0.981
Range	146.0–380.0	172.0–386.0	
AST (U/l)			
Mean ± SD	30.28 ± 8.54	24.92 ± 4.76	**0.010**
Range	19–53	17–36	
Number of patients >2 × ULN	0	0	
ALT (U/l)			
Mean ± SD	33.96 ± 25.79	26.36 ± 14.90	0.109
Range	9–114	12–71	
Number of patients >2 × ULN	1	0	
Cholesterol (mmol/l)			
Mean ± SD	4.94 ± 1.19	4.52 ± 0.84	0.147
Range	3.4–8.5	3.1–7.1	
Number of patients ≥6.2	4	1	
LDL cholesterol (mmol/l)			
Mean ± SD	2.89 ± 1.01	2.66 ± 0.65	0.357
Range	1.6–6.1	1.4–4.6	
Number of patients ≥4.1	3	1	
HDL cholesterol (mmol/l)			
Mean ± SD	1.40 ± 0.36	1.30 ± 0.36	0.331
Range	0.8–2.1	0.7–1.9	
Number of patients ≤1	4	5	
Triglyceride (mmol/l)			
Mean ± SD	1.43 ± 0.86	1.24 ± 0.56	0.345
Range	0.5–3.4	0.4–2.7	
Number of patients ≥4.5	0	0	

[a]BMI, body mass index; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ULN, upper limit of normal range.

Adverse events

Table 4 lists the adverse events. All patients reported at least one adverse event during the 6 months in both groups. No grade 3 or 4 events were reported. The incidence of any infection was similar in the sirolimus group (80%) and the standard group (88%). The most frequent infection was upper respiratory infection, and its incidence was also similar in the sirolimus group (64%) and in the standard group (80%). No clinically significant opportunistic infections were reported. Headache was more common among patients receiving sirolimus (48%) than among patients of the standard group (12%). The total number of patients with gastrointestinal adverse events was higher in the sirolimus group (84%) than in the standard group (32%). The most common gastrointestinal adverse events were mucositis (72% of the sirolimus group versus 16% of the standard group, $P = 0.0001$) and diarrhoea (36% of the sirolimus group versus 20% of the standard group, $P = 0.345$). A total of 48% patients in the sirolimus group had adverse events leading to sirolimus dose reduction, namely mucositis (16%), tooth extraction (8%), acne (4%), sirolimus trough level >10 µg/l (4%), infection (4%), leucopenia (4%) and surgery (4%).

Discussion

Sirolimus is an immunosuppressant drug with strong antiproliferative properties. It has been approved for the prevention of rejection after kidney and liver transplantation and is used in combination with other immunosuppressive drugs. The favourable effect of sirolimus in rodent models for ADPKD has prompted the initiation of clinical trials testing the efficacy of sirolimus in halting PKD progression. The potential risks of a sirolimus treatment have limited its use in transplantation and may hamper a potential therapeutic application in ADPKD patients. In this trial, however, we show that sirolimus at doses of 1–2 mg/day was well tolerated and safe in ADPKD patients.

There have been reports of nephrotoxicity and proteinuria related to sirolimus use in solid organ transplant recipients. Studies have shown an increase in urinary protein excretion in patients converted from calcineurin inhibitor-based therapy to sirolimus therapy as well as with *de novo* use of sirolimus [15–17]. The origin of sirolimus-associated urinary protein excretion, i.e. glomerular versus tubular origin, is still a subject of investigations. However, the facts that proteinuria associated with the use of sirolimus is mainly composed of albumin and, ACEi effectively reduce sirolimus induced proteinuria support the hypothesis that mTOR inhibitors increase glomerular permeability [18]. In addition to albuminuria, glomerular proteinuria is characterized by increased excretion of transferrin and IgG, two glomerular markers with different molecular weights, which allow us to distinguish selective from unselective proteinuria [19]. We analysed in detail the urinary protein excretion in 24-h urine collections and found unchanged levels of albumin, transferrin and IgG. Furthermore, the urinary excretion of the low-molecular weight proteins α_1-microglobulin and RBP,

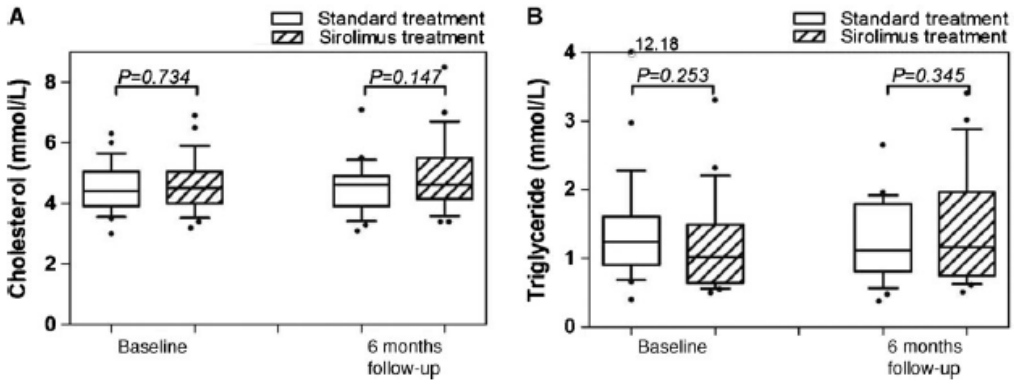

Fig. 4. Cholesterol (**A**) and triglyceride (**B**) levels were similar in both groups at baseline and at 6 months follow-up. The box indicates 50% of the observed data points between the 1st and 3rd quartiles. The line within the box represents the median. The whiskers show the data between the 10th and 90th percentiles.

characterizing tubular proteinuria [20], was unchanged by sirolimus treatment. Together, these data reveal that albuminuria is minimal in young patients affected by ADPKD. The mild albuminuria that is seen in some patients results from glomerular leaking. Sirolimus at a dosage between 1 and 2 mg/day did not significantly deteriorate glomerular proteinuria, or induce tubular proteinuria. Since a 6-month treatment with sirolimus also did not impair the GFR, sirolimus appears to have an excellent renal safety profile in ADPKD patients.

In two randomized, double-blind multicentre studies, the safety and efficacy of sirolimus compared with azathioprine (Study 1) [21] or placebo (Study 2) [22] in combination with cyclosporine and prednisone for the prevention of rejection after renal transplantation were examined. These studies compared the dose of 2 mg or 5 mg of sirolimus per day. For the dose of 2 mg/day, the following side effects compared to the control group were seen more frequently: hypercholesterolaemia, hypertriglyceridaemia, hypertension, thrombocytopenia, acne and skin rash. All side effects occurred in comedication with cyclosporine and prednisone. We found unchanged lipid levels, although there is abundant evidence that sirolimus causes an increase in serum triglyceride levels and in the levels of cholesterol, LDL and HDL [23]. In our current study, sirolimus treatment was associated with a tendency to higher values of cholesterol, LDL and triglyceride. These changes did not reach statistical significance, most probably due to type II error. Metabolic balance studies have also shown that the defect in lipid metabolism is largely dose dependent [24].

The leukocyte and platelet counts remained unchanged. However, we noted a mild but significant reduction of erythrocyte volume in patients treated with sirolimus. Several studies with sirolimus in differently combined immunosuppressive regimen as well as one study with the sirolimus derivate everolimus as a monotherapy have reported reduced MCV and microcytosis in solid organ transplant recipients [25–29]. The aetiology of the reduced MCV is not well understood. In these previous studies, patients received concomitant immunosuppressive medication to prevent organ rejection, iron supplementation and erythropoietin stimulation agents for the treatment of anaemia, and many patients had impaired kidney function, factors that may influence the morphological characteristic of erythrocytes. In our current study, we are able to ascribe this sirolimus-associated effect on MCV and MCH to the drug itself. These changes occurred at a low sirolimus trough level and were not accompanied by a decrease of leukocyte, platelet or red blood cell count, and are therefore not attributable to the well-known anti-proliferative effect of sirolimus on bone marrow colony-forming cells. Our finding of a disproportionately low MCV in comparison with unchanged red blood cell count suggests a defect in the globin synthesis. The globin protein synthesis is under tight control of eukaryotic initiation factor 2 (eIF2), and the phosphorylation of eIF2α prevents translation initiation and hence the synthesis of globin. As rapamycin causes an increase in phosphorylation of eIF2α, we speculate that the decrease in erythrocyte volume and haemoglobin content seen in our patient is due to reduced globin synthesis rather than an impaired red blood cell production [30,31].

Adverse events were transient and mild and no serious adverse events occurred in our study. The incidence of infections was similar in both groups, whereas oral ulcers and headache occurred more frequently among patients receiving sirolimus compared to patients receiving standard care. Oral adverse events, usually involving superficial ulcerations of the gingival and buccal mucosa and tongue, named aphthous mouth ulcers, have been reported in patients receiving sirolimus. Aphthous mouth ulcers occurred in 10–19% of patients receiving sirolimus in phase III clinical trials [21,22] and in up to 32% of patients switched from a calcineurin inhibitor therapy to sirolimus [32]. Notably, the comedication with corticosteroids may decrease the risk of sirolimus-associated aphthous mouth ulcers. In a cohort of patients converted to sirolimus in the absence of corticosteroids, a high incidence (up to 60%) was reported for this complication [33]. We found a 72% incidence of aphthous mouth ulcers among ADPKD patients treated with sirolimus. Various hypotheses concerning the cause of these ulcers have been generated without clear evidence. The strong anti-proliferative properties of sirolimus might function as the primary trigger for the development of these ulcers, and the absence of corticosteroids may hamper their secondary healing. Additionally, the true incidence of this adverse event might be underestimated as these mucosal

Table 4. Adverse events in the safety population from Month 0 to Month 6[a]

Category	Number of patients (%)			
	Sirolimus ($N = 25$)	Standard ($N = 25$)	Total ($N = 50$)	P-value
Any category	25 (100)	25 (100)	25 (100)	1.000
Infection				
Total	20 (80)	22 (88)	42 (84)	0.702
Upper respiratory infection, sinusitis or bronchitis	16 (64)	20 (80)	36 (72)	0.345
Urinary tract infection or pyelonephritis	4 (16)	2 (8)	6 (12)	0.667
Pharyngitis	3 (12)	2 (8)	5 (10)	1.000
Perioral	2 (8)	0	2 (4)	0.490
Pain				
Total	18 (72)	19 (76)	37 (74)	1.000
Flank pain	7 (28)	11 (44)	18 (36)	0.377
Headache	12 (48)	3 (12)	15 (30)	**0.012**
Muscoloskeletal	7 (28)	8 (32	15 (30)	1.000
Abdominal	3 (12)	2 (8)	5 (10)	1.000
Genital	2 (8)	1 (4)	3 (6)	1.000
Gastrointestinal				
Total	21 (84)	8 (32)	29 (58)	**0.0004**
Aphthous ulcer or mucositis	18 (72)	4 (16)	22 (44)	**0.0001**
Diarrhoea	9 (36)	5 (20)	14 (28)	0.345
Teeth	2 (8)	0	2 (4)	0.490
Heartburn	2 (8)	1 (4)	3 (6)	1.000
Nausea	3 (12)	0	3 (6)	1.000
Skin related				
Total	13 (52)	10 (40)	23 (46)	0.571
Acne	13 (52)	7 (28)	20 (40)	0.148
Folliculitis	1 (4)	2 (8)	3 (6)	1.000
Pulmonary or upper respiratory				
Cough	6 (24)	1 (4)	7 (14)	0.098
Renal or genitourinary				
Macrohaematuria	1 (4)	4 (16)	5 (10)	0.349
Lymphatics				
Limb oedema	3 (12)	0	3 (6)	0.235
Neurologic				
Dizziness	3 (12)	0	3 (6)	0.235
Blood and bone marrow				
Leucopenia	2 (8)	0	2 (4)	0.490
Sexual or reproductive function				
Irregular menses	2 (8)	0	2 (4)	0.490

[a]Table shows number of patients (percent). Adverse events affecting ≥5% in either group were described and graded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), version 3.0.

lesions can easily be mistaken for herpes simplex infection [34]. The incidence of headache was higher in patients receiving sirolimus. Thus far headaches were not known side effects of sirolimus treatment. Our finding has recently been validated by analysing pharmacovigilance data in other patient populations. As a consequence, the product information is being adapted on a worldwide basis to include this information.

A limitation of our study is the low number of patients and the short time of follow-up. Therefore, we cannot exclude the occurrence of rare adverse events or events occurring only after long-term treatment. However, based on experience in other patient populations treated with sirolimus, we anticipated that we could detect major side effects/adverse events in the sirolimus-treated patients with sufficient robustness in a relatively homogeneous ADPKD population in a 6-month time interval.

In conclusion, this short-term analysis reveals that sirolimus treatment is safe for ADPKD patients when the drug is used at a dosage between 1 and 2 mg/day. Treatment adherence was excellent, the renal safety profile was encouraging and the characteristic side effects of sirolimus were well manageable. The Data Safety Monitoring Board (DSMB) has therefore recommended continuation of the study. The upcoming efficacy data of this trial will establish whether sirolimus has a beneficial effect on the relentlessly progressive cyst volume growth in patients with ADPKD.

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Conflict of interest statement. None declared.

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Sirolimus Induced Phosphaturia is Not Caused by Inhibition of Renal Apical Sodium Phosphate Cotransporters

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Abstract

The vast majority of glomerular filtrated phosphate is reabsorbed in the proximal tubule. Posttransplant phosphaturia is common and aggravated by sirolimus immunosuppression. The cause of sirolimus induced phosphaturia however remains elusive. Male Wistar rats received sirolimus or vehicle for 2 or 7 days (1.5mg/kg). The urine phosphate/creatinine ratio was higher and serum phosphate was lower in sirolimus treated rats, fractional excretion of phosphate was elevated and renal tubular phosphate reabsorption was reduced suggesting a renal cause for hypophosphatemia. PTH was lower in sirolimus treated rats. FGF 23 levels were unchanged at day 2 but lower in sirolimus treated rats after 7 days. Brush border membrane vesicle phosphate uptake was not altered in sirolimus treated groups or by direct incubation with sirolimus. mRNA, protein abundance, and subcellular transporter distribution of NaPi-IIa, Pit-2 and NHE3 were not different between groups but NaPi-IIc mRNA expression was lower at day 7. Transcriptome analyses revealed candidate genes that could be involved in the phosphaturic response. Sirolimus caused a selective renal phosphate leakage, which was not mediated by NaPi-IIa or NaPi-IIc regulation or localization. We hypothesize that another mechanism such as a basolateral phosphate transporter may be responsible for the sirolimus induced phosphaturia.

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Introduction

Inorganic phosphate (Pi) is an essential nutrient involved in various life-sustaining processes such as cell metabolism and skeletal mineralization. The kidney is the major regulator of extracellular phosphate homeostasis. Phosphate is freely filtered in the glomerulus and mostly reabsorbed along the proximal tubule (PT) according to the organisms' needs to maintain a balanced serum phosphate. To date, three distinct sodium dependent phosphate cotransporter with a pivotal role for phosphate reabsorption in the kidney have been identified in the brush border membrane (BBM) of proximal tubule cells: NaPi-IIa (SLC34A1), NaPi-IIc (SLC343) and Pit-2 (SLC20A2) [1–6]. These transporters are regulated by a variety of factors and hormones including parathyroid hormone (PTH), 1,25-$(OH)_2$-vitamin D_3, and fibroblast growth factor 23 (FGF23) [4]. In the context of renal transplantation phosphate homeostasis is often disturbed and severe hypophosphatemia is a common and potential life-threatening problem during the first weeks after engraftment [7]. The preceding hyperparathyroidism and delayed reduction in FGF23 levels in patients suffering from chronic kidney disease can only partially explain reduced serum phosphate levels after kidney transplantation [8–10]. To date it is not fully understood to which extent immunosuppressive regimens further contribute to post-transplant hypophosphatemia. In fact several reports demonstrate the influence of various immunosuppressants including glucocorticoids, cyclosporine and tacrolimus on phosphate reabsorption in vivo [11–13]. Moreover, we have recently observed an aggravated and prolonged renal phosphate wasting after renal transplantation in recipients receiving sirolimus-based immunosuppression compared to patients on sirolimus-free immunosuppression [14].

Sirolimus is a potent inhibitor of the mammalian target of rapamycin (mTOR) and routinely used after solid organ transplantation to prevent rejection. mTOR has long been known for its pivotal role in regulating cell proliferation and cell growth. More recently it has been shown in the heterologous Xenopus oocyte expression system to be involved in the regulation of various solute carrier such as the creatinine transporter SLC6A8 and the renal and intestinal sodium dependent phosphate cotransporters NaPi-IIa (SLC334A1) and NaPi-IIb (SLC34A2) [15–17]. Furthermore it has been demonstrated that the stimulating effect of mTOR on NaPi-IIa and NaPi-IIb is suppressed by sirolimus [16,17]. However, detailed mechanisms how sirolimus might affect renal phosphate reabsorption remain unidentified until today. The present study aimed to elucidate the underlying mechanisms involved in sirolimus induced renal

Table 1. Blood and urine parameters from vehicle and sirolimus-treated rats after two and seven days of treatment.

	2d		7d	
	Vehicle	**Sirolimus**	**Vehicle**	**Sirolimus**
Body weight at start (g)	245.5±11.5	249.4±13.3	191.3±3.8	184.1±3.4
Body weight after treatment (g)	225.4±6.7	215.6±10.2	238.2±5.9	195.7±5.1†
Blood				
pH	7.41±0.01	7.45±0.01	7.43±0.006	7.42±0.01
PCO_2 (mmHg)	37.2±1.6	38.8±1.5	36.2±1.0	37.6±1.7
HCO_3- (mmol/l)	24.3±0.8	26.5±0.6	24.8±2.2	24.7±0.6
Na^+ (mmol/l)	142.0±1.9	143.2±1.2	140.7±0.9	140.7±0.8
K^+ (mmol/l)	4.6±0.2	3.4±0.06‡	4.3±0.3	3.4±0.2
Cl^- (mmol/l)	96±1.5	98±3.6	94.3±3.0	93.2±1.9
Calcium (mmol/l)	2.6±0.07	2.6±0.03	2.6±0.02	2.5±0.04
Phosphate (mmol/l)	3.3±0.09	2.8±0.05†	3.0±0.08	2.4±0.09‡
Creatinine (µmol/l)	17.7±0.1	19.2±1.5	22.1±3.0	17.7±0.1
Glucose (mmol/l)	10.0±0.7	10.1±0.7	8.9±0.3	15.3±1.5†
Sirolimus trough level (µg/l)	0	20.9±2.8	0	19.6±4.5
Urine				
24-h Urine/body weight (ml/g)	0.03±0.002	0.06±0.006*	0.03±0.004	0.12±0.03*
pH	6.14±0.06	6.20±0.06	6.34±0.05	6.10±0.09*
Osmolarity (mOsmol/kg)	1325±340	1584±313	1189±217	1675±250*
Creatinine Clearance (ml/min)	2.3±0.1	2.1±0.2	2.1±0.3	1.9±0.2
P^-/Creatinine (mmol/l)/(mmol/l)	12.4±0.6	17.8±0.7‡	10.4±1.1	15.8±0.9†
TmP/GFR (mmol/l)	3.0±0.1	2.5±0.06‡	2.8±0.06	2.1±0.09‡
Na^+/Creatinine (mmol/l)/(mmol/l)	13.2±2.3	18.6±4.4*	15.7±1.1	21.5±4.2*
K^+/Creatinine (mmol/l)/(mmol/l)	49.7±6.6	51.8±3.0	48.7±2.8	48.7±4.2
Cl^-/Creatinine (mmol/l)/(mmol/l)	29.2±4.1	33.2±2.7	29.2±2.7	39.6±5.9†
Mg^{2+}/Creatinine (mmol/l)/(mmol/l)	3.0±1.3	4.8±0.8*	3.6±1.2	4.9±0.4*
Ca^{2+}/Creatinine (mmol/l)/(mmol/l)	0.4±0.1	1.0±0.4†	0.5±0.1	1.7±0.8*
HCO_3^-/Creatinine (mmol/l)/(mmol/l)	0.2±0.05	0.29±0.05	0.85±0.4	1.69±0.4

Values are means ± SE, n = 6/group. A summary of blood and urine parameters from rats treated with vehicle and sirolimus for two and seven days is shown. *p<0.05. †p<0.01. ‡p<0.001.

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phosphate loss. We hypothesized that sirolimus suppresses sodium dependent phosphate reabsorption in the PT and therefore tested for a potential role of sirolimus in the regulation of renal phosphate transport across the PT mediated by NaPi-IIa, NaPi-IIc, and Pit-2.

Material And Methods

Animals

Male Wistar rats (120–150 g, Charles River, Germany) were randomly divided into four groups: groups 1 and 3 received vehicle, groups 2 and 4 sirolimus. Each group consisted of six animals and we used samples from each rat for all experiments. Daily subcutaneous injections of sirolimus (1.5 mg/kg body weight) were given for either 2 days (group 2) or seven days (group 4), whereas control animals received daily injections of vehicle for either two days (group 1) or seven days (group 3). Sirolimus (Sigma Aldrich, Germany) was dissolved in polyethylene glycol 400, 10% polysorbat 80 and 20% dimethylacetamid. Vehicle consisted of the three solvents. Rats were maintained on a standard rodent chow containing 0.8% phosphate (Kliba AG, Kaiseraugst, Switzerland) and had access to drinking water ad libitum. All animals were placed in individual metabolic cages for three and eight days, respectively, allowing a 24 hour adaption period to the metabolic cage environment. Food and water consumption, body weight, stool and urine output were monitored daily. Urine samples were collected daily under mineral oil. All animal experiments were performed according to national and international guidelines and laws of animal welfare. Protocols were approved by the local veterinary authorities (Veterinaeramt Zurich 11/2010, Bundesministerium für Wissenschaft und Forschung Österreich 66009/4-II/10b/2010).

Blood and Urine Analysis

At the end of the experiments rats were anesthetized by inhalation of Isoflurane/air and heparinized arterial blood was collected from the tail artery and immediately analyzed for pH, blood gases, and electrolytes on a Radiometer ABL 800 Flex blood gas analyzer (Radiometer, Copenhagen, Denmark). Serum creatinine, serum phosphate and serum glucose concentrations were determined using the clinical chemistry analyzer Piccolo Xpress (LabForce, Nunningen, Switzerland). Vitamin D plasma levels were determined using the 1,25 Dihydroxy Vitamin D RIA (Immunodiagnostic Systems Ltd., Baldon, UK) according to the

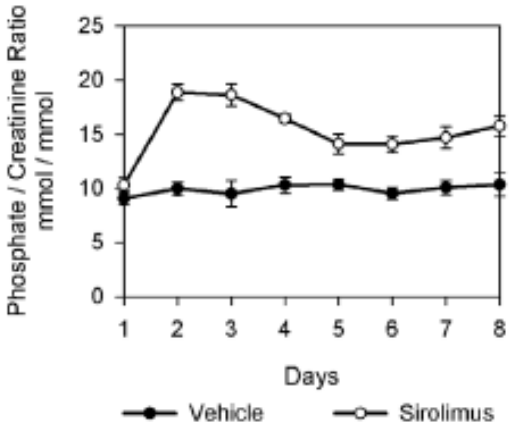

Figure 1. Effect of sirolimus on renal phosphate excretion. Trajectories of urinary phosphate/creatinine ratios of sirolimus and vehicle treated rats. Sirolimus caused statistically significant increased urinary phosphate excretion 24 hours after the first sirolimus injection (day 2) until after seven sirolimus injection (day 8, n = 6 for each group).
doi:10.1371/journal.pone.0039229.g001

manufacture's protocol. The levels of intact fibroblast growth factor 23 (intact FGF23, Kainos Laboratories Inc., Tokyo, Japan) intact parathyroid hormone (intact PTH, Immunotopics Inc., San Clemente CA, USA) and soluble klotho (Immuno-Biological Labratories Co., Minneapolis, USA) were measured by a two-site enzyme-linked immunosorbent assay in rodent serum samples, according to the manufacturer's protocol. Inter-assay and intra-assay coefficients of variation were below 5% for all laboratory analyses. Creatinine clearance was calculated from the measured values. Sirolimus blood levels were measured using HPLC-mass spectrometry [18]. For urinary pH, PCO_2 and calculated HCO_3^- measurement urine was aspirated from the collectors (urine was collected under mineral oil) into syringes and injected into the blood gas analyzer. Urinary creatinine was analyzed using the Jaffè method [19]. Urinary phosphate was determined by endpoint method with sample blanking [19,20]. Urinary K^+, Na^+, Cl^-, Ca^{2+} and Mg^{2+} concentrations were measured using a chemistry analyzer (Cobas Integra 800, Roche, Urinary osmolarity was determined using a One-Ten Osmometer (Fiske Associates, Norwood, Massachussettes, USA) by examination of freezing-point depression. A test for low molecular weight proteinuria was performed. Urine samples were normalized for creatinine and samples containing 10 mg creatinine were then solubilized in Laemmli sample buffer and SDS-PAGE was performed on a 15% polyacrylamid gel. Colloidal coomassie blue staining was performed for 1h at room temperature. After destaining for 2h, the gel was photographed and dried. Five micrograms of BSA served as a positive control.

RNA extraction and semi-quantitative real time qRT-PCR

Total RNA from kidneys was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland). Snap-frozen kidneys were homogenized in RLT buffer (Qiagen)

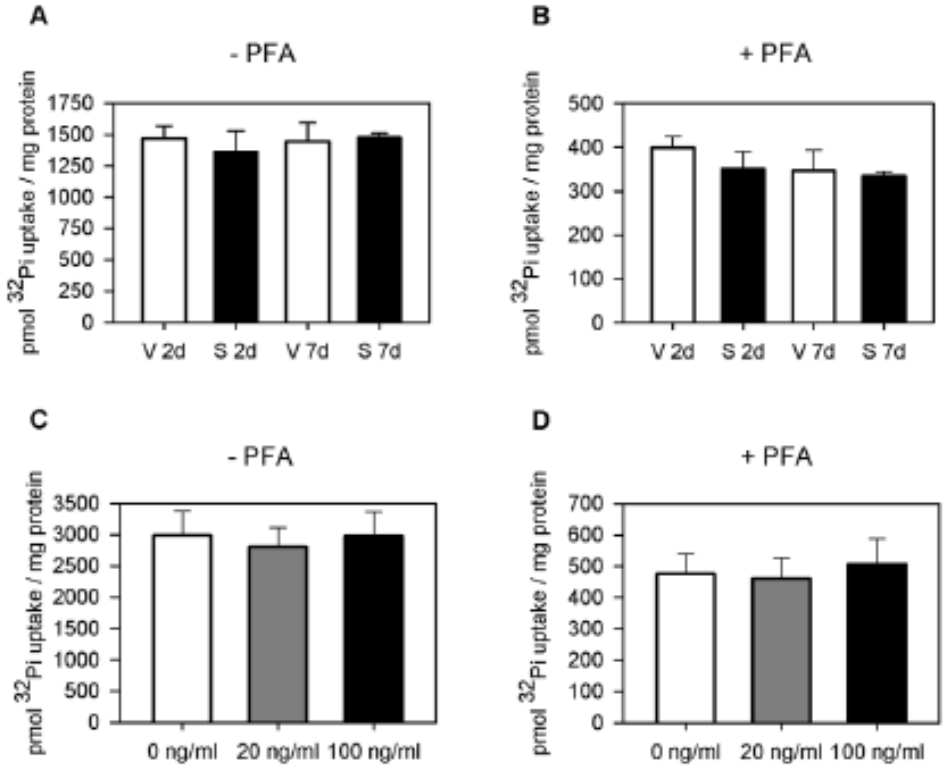

Figure 2. Sirolimus has no effect on apical renal phosphate transport. Effect of Sirolimus treatment for two and seven days on BBM sodium-dependent phosphate uptake in absence and presence of phosphonoformic acid (6 mM, PFA), an inhibitor of SLC34 phosphate cotransporter. Sirolimus did not alter ^{32}Pi-uptake in absence (**a**) or presence (**b**) of PFA after two and seven days of sirolimus treatment (n = 6 each group). Incubation of BBMV from untreated rats with 20ng/ml and 100ng/ml sirolimus did not change Na^+-dependent phosphate fluxes in the absence (**c**) or presence (**d**) of PFA.
doi:10.1371/journal.pone.0039229.g002

Table 2. Serum values of phosphate regulatory hormones from vehicle and sirolimus-treated rats after two and seven days of treatment.

	2d		7d	
	Vehicle	Sirolimus	Vehicle	Sirolimus
PTH (pg/ml)	402.9±37.0	177.1±35.2†	333.9±43.6	192.5±30.5*
FGF23 (pg/ml)	259.0±15.4	267.3±21.0	249.3±19.7	144.2±12.2‡
Klotho (pg/ml)	466.5±134.6	592.6±164.5	1110.8±264.1	809.1±177.3
1,25 Dihydroxycholecalciferol (pg/ml)	123.0±10.5	154.5±9.1	130.5±12.2	177.6±19.9

Values are means ± SE, n = 6/group. A summary of blood and urine parameters from rats treated with vehicle and sirolimus for two and seven days is shown. *p<0.05. †p<0.01. ‡p<0.001.

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supplemented with ß-mercaptoethanol to a final concentration of 1%. Subsequently, 200 µl of each homogenate were used for total RNA isolation according to the manufacturer's protocol. DNAse digestion was performed using the RNase-free DNAase set (Qiagen, Hilden, Germany). Quality and concentration of the isolated RNA preparations were analyzed spectroscopically using the 2100 Bioanalyzer (Agilent Technologies) and the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA samples were diluted to a final concentration of 100 ng/µl and 3 µl were used for cDNA preparation using the TaqMan Reverse Transcriptase Reagent Kit (Applied Biosystems/Roche, Foster City, CA). Primers and probes (Microsynth, Balgach, Switzerland) of all genes of interest were designed using Primer Express Software (v.2.0.; Applied Biosystems), and primers were tested by PCR with kidney cDNA and always resulted in a single product of the expected size (data not shown). Sequences of primers and probes are listed in Data S1. Real-time PCR reactions were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems). Briefly, 3 µl cDNA, 0.8 µl of each primer (25 µM) and 0.4 µl of labeled probe (5 µM), 5 µl RNase-free water, 10µl TaqMan Universal PCR Master Mix added to a final volume of 20 µl. Reactions were run in 96-well optical reaction plates using the Prism 7500 fast Real-Time PCR cycler. Cycling conditions were set to one cycle for 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C (10s) and 60°C (1 min) with auto ramp time. All reactions were run in triplicate, and one negative control without addition of the multiscribe reverse transcription enzyme was included for each sample. The relative abundance of target mRNA was calculated to a reference mRNA (hypoxanthine-guanine-phosphoribosyltransferase; HPRT). Relative expression levels were calculated as $R = 2^{[Ct(HPRT) - Ct(gene)]}$, where Ct is the cycle number at which the fluorescence intensity is above background levels (threshold).

Brush border membrane vesicle preparation and phosphate uptake experiments

Frozen kidneys were used to prepare total membrane proteins and brush border membrane vesicle (BBMV) using the Mg^{2+} precipitation technique as previously described [21,22]. The phosphate transport rate into BBMV was measured in freshly prepared BBMV at 25°C in the presence of inward gradients of 100 mM NaCl or 100 mM KCl and 0.1 mM K-phosphate. The substrate Pi was made with 0.125 mM K2HPO4 and 32P (1µCi/ml) to give a final concentration 0.1 mM close to the expected apparent Km for Pi for Na+-dependent transport in renal BBMV. The stop solution contained 100 mM Mannitol, 5 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM Pi. Na+-dependence was established by incubating BBMV in solutions in which KCl replaced NaCl equimolarly. Phosphate uptake was determined after 60 s, representing initial linear conditions, and after 120 min, to determine the equilibrium values. In order to distinguish between Na+-dependent Pi uptake mediated by SLC34 family members (e.g., NaPi-IIa and NaPi-IIc) and other Na+-dependent phosphate transporters such as SLC20 family members (e.g., Pit-1 and Pit-2), we used trisodium phosphonoformic acid (PFA, final concentration 6 mM) added to the same solution with 107mM NaCl. PFA is known to be an inhibitor of BBMV phosphate uptake since decades [23,24] but has previously been shown to have a higher selectivity for type II (i.e. NaPi-IIa and NaPi-IIc, SLC34) than type III (i.e. Pit-2, SLC20) phosphate transporters at these concentrations [2,25–27]. Total protein concentration was measured using the Bio-Rad Protein Assay kit, Bio-Rad, Hercules, CA, USA. BBMV were stored at −80°C until further use.

Immunoblotting

Ten micrograms of renal BBM protein or 35µg of total membrane protein was solubilized in Laemmli sample buffer and SDS-PAGE was performed on 8% polyacrylamid gels. Proteins were transferred electrophoretically from gels to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). After blocking with 5% milk powder in tris-buffered saline containing 0.1% tween 20 for 60min, the blots were incubated with the respective primary antibodies: rabbit polyclonal anti-NaPi-IIa (1:6,000) [28], rabbit polyclonal anti-NaPi-IIc (1:10,000) [29], rabbit polyclonal anti-Pit-2 (1:3,000) (kindly provided by V. Sorribas, University of Zaragosa, Spain), rabbit polyclonal anti NHE3 (1:5,000)(generated by immunization of rabbits with a peptide linked to KLH, Pineda antibody service, Berlin, Germany) rabbit polyclonal anti-klotho (Abcam, Cambridge, UK) and mouse monoclonal anti-β-actin antibody (42kD; Sigma, St. Louis, MO; 1:5,000) overnight at 4°C. After washing and subsequent blocking, blots were incubated with the secondary antibody (donkey anti-rabbit or sheep anti-mouse antibodies linked to horseradish peroxidase 1:10,000, (GE Healthcare, Little Chalfont, Buckinghamshire, UK) or goat anti-rabbit antibody 1:5,000 linked to alkaline phosphatase (Promega, Madison, WI) for 1h at room temperature. Antibody binding was detected with the Immobilon western chemiluminescence kit (Millipore, Billerica, MA), using the DIANA III-chemiluminescence detection system (Raytest, Straubenhardt, Germany). All images were analyzed using appropriate software (Advanced Image Data Analyzer, Raytest) to calculate the protein of interest/ß-actin ratio.

Immunohistochemistry

Anesthetized rats were fixed by vascular perfusion through the left ventricle. The thorax was opened and the fixative was

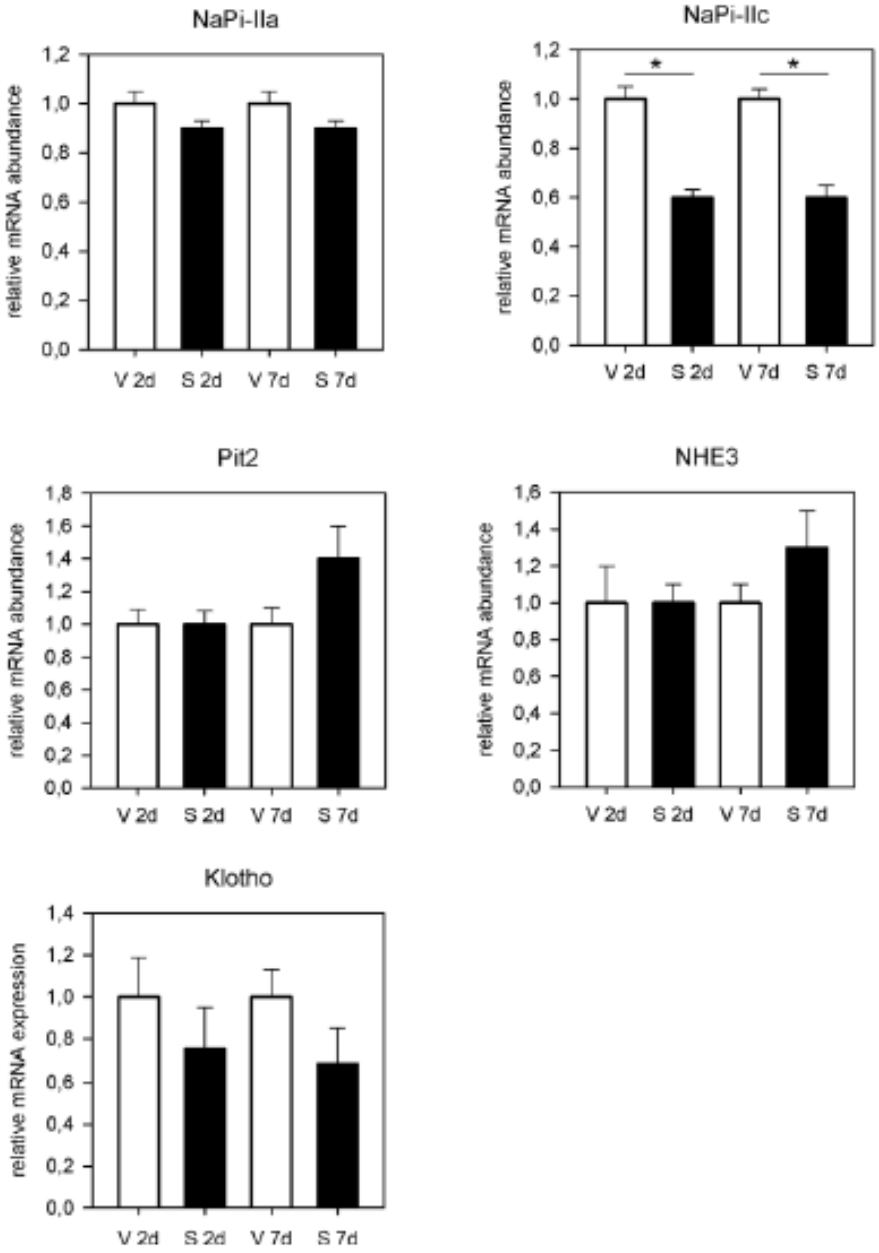

Figure 3. Sirolimus has no effect on renal phosphate transporter mRNA abundance. Results of the semiquantitative RT-qPCR for NaPi-IIa, NaPi-IIc, Pit-2, NHE3 and klotho after two and seven days of sirolimus treatment. mRNA abundance for NaPiHa, klotho, Pit2 and NHE3 was not different between groups after two and seven days of sirolimus treatment. NaPi-IIc mRNA abundance was significantly lower in sirolimus treated animals after two and seven days. * p<0,05.
doi:10.1371/journal.pone.0039229.g003

manually injected through a 50 ml syringe. The fixative consisted of 3% paraformaldehyde, 0.05% picric acid in 0.1M cacodylate buffer (pH 7.4; containing 3 mM $MgCl_2$ and adjusted to 300 mosmol/l with sucrose) and 4% hydroxyethyl starch in saline (HAES sterile; Fresenius, Stans, Switzerland). After ten minutes the fixative was washed out by perfusion with PBS. Kidneys were then removed and stored in PBS overnight at 4°C. Coronal slices of fixed kidneys were then frozen in liquid propane and cooled with liquid nitrogen and stored −80°C. Serial sections, 5 µm thick, were cut at −20°C on a cryomicrotome (CM 1850-1-1, Leica Microsystems, Nussloch, Germany) mounted on thermo scientific superfrost plus glass slides (Thermo Fischer Scientific Inc, Braunschweig, Germany), thawed, and kept in cold PBS until further processing for staining. Before immunofluorescence staining, sections were pretreated with blocking solution (Normal Goat Serum 10% in PBS with 0.5% bovine serum albumin,0.04%Na-Azide) for 60 min at room temperature. After blocking sections were incubated with the primary antibody overnight at 4°C either with a rabbit anti-rat antiserum against the NaPi-IIa protein [28] diluted 1:1,000 or with an immunopurified rabbit anti-mouse NaPi-IIc [29] diluted 1:1,500, or a rabbit anti-rat Pit-2 [2] diluted 1:250. All primary antibodies were diluted in

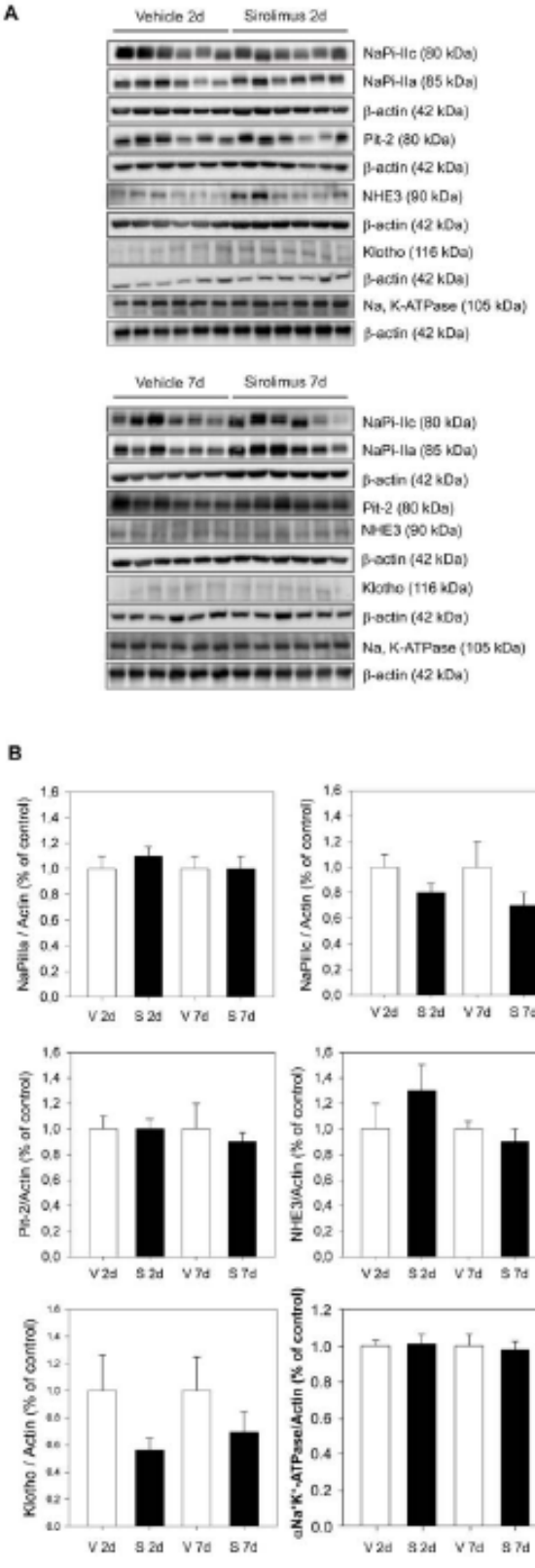

Figure 4. Sirolimus does not alter renal phosphate transporters. Sirolimus treatment for two and seven days does not alter protein expression levels of NaPi-IIa, NaPi-IIc, Pit-2, klotho and NHE3 in the brush border membrane. Brush border membranes or total membrane protein were prepared from kidneys of sirolimus and vehicle injected rats (n = 6) and 10 µg of brush border membranes or 35 µg of total membrane protein were loaded per lane for immunoblotting. **a** Membranes were tested for NaPi-IIa, NaPi-IIc, Pit-2, NHE3, and the αNa+/K+-ATPase subunit and stripped for reprobing with ß-actin to control for loading. **b** Densitometric analysis of all immunoblots with the appropriate software was performed and bands of the proteins of interest were normalized against ß-actin and the respective vehicle groups. Sirolimus treatment for two and seven days did not change the abundance of NaPi-IIa, NaPi-IIc, Pit-2, klotho and NHE3 compared to vehicle.
doi:10.1371/journal.pone.0039229.g004

PBS with 0.5% bovine serum albumin. Sections were then rinsed three times with PBS and covered for 90 minutes at room temperature with Alexa Fluor 555 goat–anti-rabbit IgG (1:1,000, Invitrogen), FITC–phalloidin (Molecular probes, Eugene, OR, USA, 1:200), and 4,6-diamidino-2-phenylindole (DAPI; Sigma, St Louis, MO) diluted 1:500. Finally, the sections were rinsed three times with PBS, cover slipped using DAKO-Glycergel (Dakopatts) containing 2.5% 1,4-diazabicyclo [2.2.2] octane (Sigma) as a fading retardant. Immunohistochemistry images were acquired with a Leica DFC490 charged-coupled device camera attached to a Leica DM 6000 fluorescence microscope (Leica, Wetzlar, Germany) using equivalent camera parameters for kidneys sections stained with the same primary antibody. Pictures were processed using Adobe Photoshop (overlays).

Statistical analysis

All data are summarized as mean ± SE and were analyzed using the unpaired Student's t- test with p values ≤0.05 considered as statistically significant. Urinary phosphate/creatinine ratio course throughout the experiment was analyzed using a mixed linear model. We calculated the correlation coefficients for magnesium, calcium, sodium, chloride and potassium in correlation to phosphate in urine. SAS V9.2 for windows was used as statistical software (2008 SAS System Inc., Cary, NC, USA).

Results

Animal model, blood and urine parameter

Mean body weight was similar in all groups at the beginning of the experiment and after two days of treatment with sirolimus but was significantly lower in sirolimus treated animals after seven days of treatment (238.2 g ±5.9 g vs 195.7 g ±5.1 g, p = 0.0003) (table 1). Sirolimus treatment with 1.5 mg/kg/body weight for two and seven days resulted in plasma trough levels (µg/l) of 20.9±2.8 at day 2 and 19.6±4.5 at day seven. The treatment with the mTOR inhibitor did not change acid-base homeostasis as determined by arterial blood gas and urine analysis. Blood glucose levels did not differ between groups after two days but sirolimus treated animals exhibited higher serum glucose levels at day seven. Sirolimus treatment for two and seven days had no effect on creatinine clearance and serum creatinine levels. Sirolimus treatment was associated with polyuria indicated by a significantly higher 24-h urine/body weight (ml/g) after two and seven days (0.03±0.002 vs 0.06±0.006, p = 0.004, and 0.03±0.004 vs 0.12±0.03 p = 0.03, respectively). Accordingly, urine osmolarity (mOsmol/kg) was significantly decreased in sirolimus treated animals after seven days but was not significantly decreased after two days (1325±340 vs 1584±313, p = 0.2 and 1189±217 vs 1675±250 p = 0.02, respectively). Serum Na^+, Cl^-, Ca^{2+} showed no significant changes, however, serum K^+ was significantly lowered in sirolimus treated rats after two days (4.6±0.2 vs 3.4±0.06, p = 0.0001). Urinary Na^+/creatinine ratios ((mmol/l)/(mmol/l)) were significantly higher after two and seven days

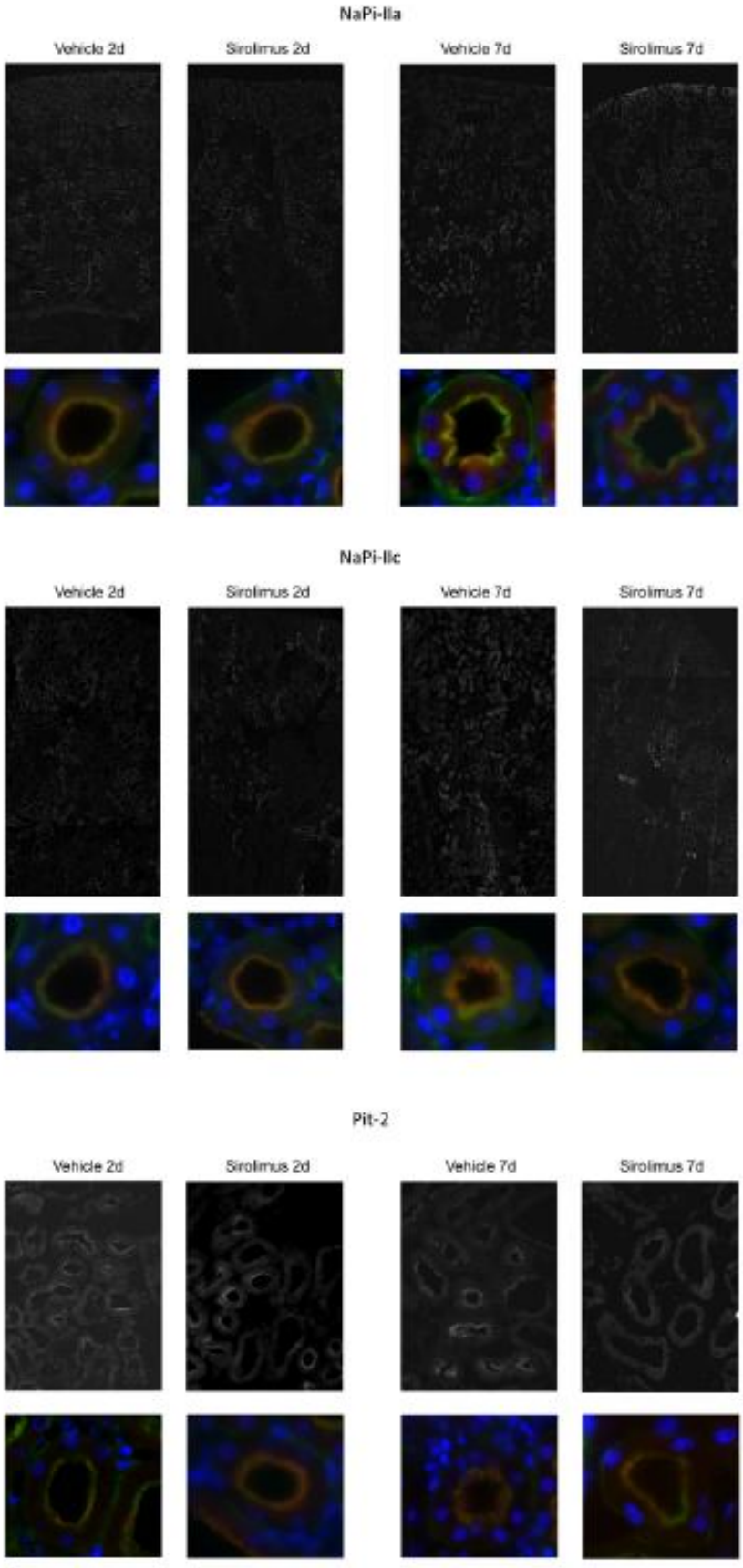
NaPi-IIa
Vehicle 2d
Sirolimus 2d
Vehicle 7d
Sirolimus 7d
NaPi-IIc
Vehicle 2d
Sirolimus 2d
Vehicle 7d
Sirolimus 7d
Pit-2
Vehicle 2d
Sirolimus 2d
Vehicle 7d
Sirolimus 7d

Figure 5. Sirolimus has no effect on localization of renal phosphate transporters. Effect of sirolimus treatment for two and seven days on NaPi-IIa, NaPi-IIc and Pit-2 localization. NaPi-IIa, NaPi-IIc or Pit2 staining (red) was observed in the BBM of early proximal tubules, and colocalized with β-actin as a marker of the BBM (green) as indicated by the yellow overlay. Nuclei were stained with DAPI (blue). No difference was observed between animals treated with vehicle or sirolimus for either 2 or 7 days (n = 5 per group). Original magnification 630 x.
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treatment (13.2±2.3 vs 18.6±4.4, p = 0.02 and 15.7±1.1 vs 21.5±4.2, p = 0.02). Cl^-/Creatinine ratio ((mmol/l)/(mmol/l)) was significantly higher after seven days (29.2±2.7 vs 39.6±5.9, p = 0.003) but was not different after two days. Mg^{2+}/Creatinine ratios and Ca^{2+}/Creatinine ratios ((mmol/l)/(mmol/l)) were significantly higher after two and seven days (magnesium 3.0±1.3 vs 4.8±0.8, p = 0.02 and 3.6±1.2 vs 4.9±0.4, p = 0.04; calcium 0.4±0.1 vs 1.0±0.4, p = 0.006 and 0.5±0.1 vs 1.7±0.8, p = 0.01). Only very weak overall correlations of urinary phosphate with magnesium, sodium and chloride, calcium and potassium were found (Data S1). Whereas sirolimus treatment for two days did not change urinary pH, animals treated with sirolimus for seven days excreted a slightly more acidic urine compared to vehicles (6.34±0.05 vs 6.10±0.09, p = 0.03). However, the bicarbonate excretion into the urine remained unchanged between groups as determined by HCO_3^-/creatinine ratio. Urinary protein excretion was similar between all groups excluding proteinuria (data not shown). Urinary glucose/creatinine ratio ((mmol/l)/(mmol/l)) was significantly higher only after seven days (0.3±0.1 vs. 135.4±113.6, p = 0.03).

Functional studies of phosphate homeostasis

Urine phosphate/creatinine ratio was higher in sirolimus treated rats beginning at 24 hours after the first injection and continued to be higher throughout the whole experiment for two and seven days (figure 1, p<0.0001) Furthermore, serum phosphate (mmol/l)) was significantly lower in sirolimus treated rats after two and seven days (3.3±0.09 vs 2.8±0.05, p<0.01 and 3.0±0.08 vs 2.4±0.09, p<0.001) (table 1). Moreover Tmp/GFR (mmol/l) was significantly lower in sirolimus treated rats after two and seven days (3.0±0.1 vs 2.5±0.06, p<0.001 and 2.8±0.06 vs 2.1±0.09, p<0.001, respectively). While serum-PTH (pg/ml) was lower in sirolimus treated rats after two and seven days (403±37 vs 177±35, p = 0.001 and 334±44 vs 193±31, p = 0.02), FGF 23 levels (pg/ml) were unchanged after two days but significantly lower in sirolimus treated rats after seven days (259±15 vs 267±21 p>0.05 and 249±20 vs 144±12, p = 0.001). Soluble klotho serum levels and vitamin D_3 serum levels were not significantly affected by sirolimus treatment (table 2). BBMV ^{32}Pi uptakes in the absence and presence of phosphonoformic acid (PFA) were similar in sirolimus and vehicle injected rats after two and seven days and revealed no different characteristics in phosphate influx (figure 2). Moreover, incubation of BBMV from untreated rats with two different sirolimus concentrations, 20ng/ml and 100ng/ml respectively, during the preparation and uptake procedure had no effect on Na^+-dependent phosphate fluxes (figure 2).

Renal phosphate regulation on the transcriptional level

Results from semiquantitative RT-qPCR for NaPi-IIa, NaPi-IIc and Pit-2 revealed only minor changes in mRNA abundance for these sodium dependent phosphate cotransporters in the PT of kidneys from sirolimus treated rats (figure 3). In detail mRNA abundance for NaPi-IIa, Pit2 as well as for klotho was not different between groups after two and seven days of sirolimus treatment. NaPi-IIc mRNA abundance was significantly lower in sirolimus treated animals after two and seven days (1±0.05 vs 0.6±0.05, p = 0.0003 and 1±0.04 vs 0.6±0.0, p = 0.0002). Furthermore NHE3 mRNA levels were not affected by sirolimus treatment. Our microarray data showing no significant difference between NaPi-IIa, NaPi-IIc and NHE3 confirm these RT-qPCR results. Microarray analysis was performed on kidney samples from the same animals. In total 154 features were identified as significant differentially expressed when comparing the gene expression profiles of rat kidneys after seven days of treatment with either sirolimus or vehicle with a fold change over 1.5, resulting in 139 down-regulated and 15 up-regulated genes in the sirolimus group. According to PANTHER classification down-regulated transcripts belong to response to stimulus, metabolic processes, immune system, transport, and signal transduction. Up-regulated transcripts belong also to response to stimulus, immune system, and transport but also to blood coagulation and regulation of vasoconstriction. Interestingly transcriptome analyses revealed several potential candidate genes that may be involved in tubular phosphate transport. SLC17A4, a putative sodium-dependent phosphate transporter protein was significantly down regulated in sirolimus treated rats compared to vehicle [30].

Renal phosphate regulation on the protein level

Sirolimus treatment for two and seven days did not lead to a significant alteration in protein expression levels as determined by immunoblotting for NaPi-IIa, NaPi-IIc, Pit-2, klotho, NHE3, and the alpha subunit of the Na^+/K^+-ATPase (figure 4). The relative protein abundance of NaPi-IIa, NaPi-IIc and Pit-2 remained similar between groups after two and seven days. Additionally, immunostaining for NaPi-IIa, NaPi-IIc, and Pit-2 did not reveal any difference in transporter distribution and respective localization at the apical surface of proximal tubule cells between sirolimus and vehicle treated rats after two and seven days (figure 5).

Discussion

This study demonstrates that sirolimus causes hypophosphatemia and hyperphosphaturia as observed in patients treated with this immunosuppressant [14]. The rats receiving sirolimus also recapitulated other side effects such as hyperglycemia and glucosuria often observed under sirolimus treatment [31]. Our data indicate that the sirolimus induced urinary phosphate wasting is not caused by dysfunction of the three currently known phosphate transporters located in the BBM of the proximal tubule. Our results furthermore indicate an intact physiological feedback mechanisms of the phosphate regulating hormones PTH, 1,25 dihydroxycholecalciferol, FGF23 and its cofactor klotho to counterbalance the renal phosphate loss.

Sirolimus treated rats did not develop signs of general renal failure or a more generalized Fanconi-like dysfunction of the PT as indicated by the absence of bicarbonaturia or proteinuria. Furthermore NHE3 mRNA as well as protein abundance was not different between groups. The dose of sirolimus applied here is comparable to that used in similar animal studies [32–34].

Several lines of evidence support the conclusion that sirolimus did not induce phosphaturia by primarily acting on BBM phosphate transporters despite a significantly lower mRNA abundance of NaPi-IIc. i) the rates of Na^+-dependent phosphate uptake into BBMV was not affected by treatment of rats with

sirolimus or acute incubation in vitro with high concentrations of sirolimus, ii) mRNA and protein expression of NaPi-IIa and Pit-2 were unaffected by sirolimus treatment. Solely mRNA expression of NaPi-IIc was significantly down regulated after two and seven days. However, sirolimus did not alter protein expression of NaPi-IIc iii) the subcellular localization of these three transporters with predominant localization in the BBM of the early PT was not altered, iv) microarray analysis of renal transcripts did not detect changes in any mRNA related to the known phosphate transporters or proteins known to be involved in their expression or activity, and v) hormonal changes are consistent with compensatory adaptations but not with causing phosphaturia.

Our results are in apparent contradiction with two recent reports suggesting that the mTOR pathway may regulate the renal and intestinal phosphate cotransporters NaPi-IIa and NaPi-IIb [16,17] Both studies describe the in vitro stimulation of NaPi-IIa and NaPi-IIb induced phosphate transport in Xenopus oocytes by coexpression of the mTOR kinase and the reversion of the stimulatory effect by rapamycin. In contrast, our study is performed in vivo and fails to detect any impact on the renal NaPi-IIa cotransporter. Moreover, Moz et al demonstrated that the calcineurin A beta subunit is involved in the regulation of NaPi-IIa in vivo and required for the normal adaptation of NaPi-IIa expression in response to changes in dietary phosphate intake [35]. The requirement of calcineurin A beta may explain the phosphaturia observed in calcineurin inhibitors such as tacrolimus. However, sirolimus does not directly affect calcineurin A beta.

It has been previously shown that klotho may exert some phosphaturic effects independently from FGF23 [36]. However sirolimus induced phosphaturia in our in vivo rat model did not alter renal klotho mRNA and protein expression or serum levels.

We also performed whole kidney transcript anaylasis using microarrays to detect candidate targets of sirolimus that may participate in the induction of phosphaturia. However, the analysis of significantly altered transcripts did not reveal any genes with a clear relation to renal phosphate transporters or their regulation. Microarray data indicated downregulation of SLC17A4. This transporter belongs to the SLC17 superfamily of transporters with subgroups of urate and vesicular gluatamte transporters [37]. Initially several transporters from this family have been assigned as phosphate transporters due to the induction of phosphate transport when heterologously expressed in Xenopus oocytes [38,39]. More recent experiments identified urate or glutamate as physiological substrates [37]. However, the substrates of SLC17A3 have not been reported to date and its exact expression pattern not reported. None of the altered transcripts has been connected to renal phosphate handling to date. Further analysis of transcripts affected by sirolimus treatment is required to understand their biological significance.

The direct target(s) of sirolimus causing phosphaturia remain(s) elusive. Our results suggest that direct dysregulation of BBM phosphate transporters can be excluded and that other mechanisms must be considered. Two alternative targets might be either basolateral PT phosphate exit pathways or phosphate transport mechanisms located in the more distal nephron. Earlier studies suggested the presence of phosphate absorbing mechanisms in the distal tubule, however, their functional significance, regulation, molecular identity, or even existence have remained elusive to date [40,41]. Completion of phosphate absorption in the PT requires (a) exit pathway(s) across the basolateral membrane. Only few data exist analyzing functional properties of the basolateral phosphate transport mechanisms but the molecular identity of basolateral phosphate transport proteins is unknown to date [42,43].

Conclusion

Sirolimus induces renal phosphate wasting and the absence of changes in the expression of all known renal phosphate transporters and normal BBM phosphate transport rates suggest an alternative mechansim. Moreover, the regulation of the major known phosphate regulating hormones, PTH, 1,25 Dihydroxycholecalciferol, FGF23 in response to hypophosphatemia was intact and suggests rather compensatory adaptation. Sirolimus might affect other mechanisms that could contribute to overall renal phosphate handling such as either basolateral exit pathways for phosphate in the PT or other elusive transport pathways in downstream nephron segments. Clearly, further studies are needed to unravel the molecular mechanisms causing sirolimus induced phosphaturia.

Supporting Information

Data S1 Supporting Information
(DOCX)

Author Contributions

Conceived and designed the experiments: JB CAW RO. Performed the experiments: SA BB IP MH NM. Analyzed the data: MH JW AK CAW RO. Contributed reagents/materials/analysis tools: AS CAW RO. Wrote the paper: MH CAW RO.

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